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B I O C H E M I C A L A S P E C T S
O F
O L F A C T O R Y M E C H A N I S M S

A T H E S I S
submitted in fulfilment of
the requirements for the degree
of
DOCTOR OF PHILOSOPHY
of the
University of Warwick
by
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Department of Molecular Sciences
November, 1976.

THE FROG

What a wonderful bird the frog are
When he stand he sit almost;
When he hop he fly almost.
He ain't got no sense hardly;
He ain't got no tail hardly either.
When he sit, he sit on what he ain't got almost.

ANON.

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ACKNOWLEDGMENTS

I wish to express my gratitude to research Supervisor, Dr. G. H. Dodd, for his help and criticism.

I also wish to thank Professors V. M. Clark and K. J. Jennings for allowing me to use the facilities in the Department of Molecular Sciences.

My gratitude is extended to Mr. T. M. Poynder for the loan of his applicator and for his co-operation in some of the photoaffinity labelling experiments which were carried out in his laboratory at the University College of London. I appreciate his friendship and the assistance he gave me on electrophysiological techniques.

My thanks go to the technical staff of the Department of Molecular Sciences for their help in making an olfactometer.

I would like to thank Carol and David for reading this manuscript, and Ahmet for his help with the diagrams.

I would also like to thank Mrs V. Gladman for typing the manuscript.

A Scholarship from the Ministry of Education of the Turkish Government and the grant from the British Council are greatly acknowledged.

SUMMARY

Electron microscopic examination of intact olfactory epithelia in different animals yielded the same pattern of morphological organisation of the cellular organelles.

Attempts have been made to isolate cilia from the primary olfactory neurons in order to study the molecular mechanisms involved in olfaction. The results indicated that the purity and quantity of the ciliary preparation obtained by current membrane fractionation methods was not suitable for biochemical investigations. Also ciliated columnar respiratory cells have been found in the olfactory mucosa of sheep. This feature presents difficulties for future neuronal membrane isolation attempts.

Two basic problems in olfaction, coding and transduction have also been studied in this thesis.

Chemical modification methods have been employed to study the coding mechanism at the peripheral level in the frog. With a variety of group specific protein reagents, proteolytic enzymes and photoaffinity odorants, it has been possible to specifically modify olfactory receptors.

The non-penetrant sulfhydryl reagent, mersalyl irreversibly inhibited the EOG responses to odorants. This effect was found to be

prevented by n-amyl acetate. Application of this odorant in solution before, during and after the treatment of the mucosa with mersalyl resulted not only in protection of the responses to n-amyl acetate, but also to other odorants as well. Responses to some odorants did not show any recovery providing evidence for the occurrence of different receptor sites.

To investigate the possible involvement of cAMP in the olfactory transduction mechanism, a number of chemicals were applied to the frog's olfactory mucosa. Phosphodiesterase inhibitors and membrane-permeable cyclic AMP derivatives decreased the EOG responses.

One contribution of this thesis to the field of olfaction has been to show that olfaction has biochemical features common to other receptor systems.

ABBREVIATIONS

| | |
|----------------|--|
| ADP | Adenosine 5'-diphosphate |
| ATP | Adenosine 5'-triphosphate |
| BSA | Bovine serum Albumin |
| cAMP | Adenosine-3',5'-cyclic monophosphate |
| CETAB | Cetyltrimethylammonium bromide |
| cGMP | Guanosine-3',5'-cyclic monophosphate |
| DTT | Dithiothreitol |
| DTNB | 5,5'-dithiobis-(2-nitrobenzoic acid) |
| EDTA | Ethylenediamino-tetra-acetic acid |
| EOG | Electro-Olfacto-Gram |
| FDNB | 1-Fluoro-2,4-dinitrobenzene |
| GTP | Guanosine 5'-triphosphate |
| IR | Infrared |
| K _D | Dissociation constant |
| MW | molecular weight |
| Nbf-Cl | (4-chloro-7-nitrobenzofurazan) |
| NEM | N-ethyl maleimide |
| NMR | Nuclear Magnetic Resonance |
| OD | Optical density |
| RO 20-1724 | 4(3-Butoxy-4-methoxybenzyl)-2-imidazolidionine |
| SDS | Sodium dodecylsulphate |
| SQ20009 | [1-ethyl-4-isopropylidenehydrazino)-1H-Pyrazolo -(3,4-b) pyridine-5-carboxylic acid, ethylester, HCl] |
| u.v. | ultraviolet |
| λ | wavelength |

Other abbreviations are explained in the text.

CHAPTER 1

INTRODUCTION

1.1. THE SENSE OF SMELL, OLFACTION

Many aspects of cellular behaviour indicate that cells have specific characteristics such that they belong to a certain tissue, to a certain species and to a certain individual. Some cells are highly specialised with respect to both sensitivity to external influences (or stimuli) and the way in which they respond. In the specialised chemoreceptor organs, such as the chemoreceptor of the carotid bodies or the olfactory sense organ (olfactory mucosa), the cells are able to transduce the stimulus into a coded message that provides the central nervous system with information about the strength and the nature of the stimulating compound. For many animal groups the information obtained by vision is the most important, while in others information about the chemical nature of the external environment is more essential.

The significance of the sense of smell in the life of different vertebrates seems to be closely related to differences in the phylogenetic development of the brain. Ottoson and Shepherd pointed out that in lower vertebrates the rudimentary cerebral cortex is largely olfactory in function and the olfactory stimuli consequently have a great influence on the life of these animals (Fig. 1). In parallel with this, it was said that the predominance of the sense of smell is lost and vision and audition become more significant (Ottoson

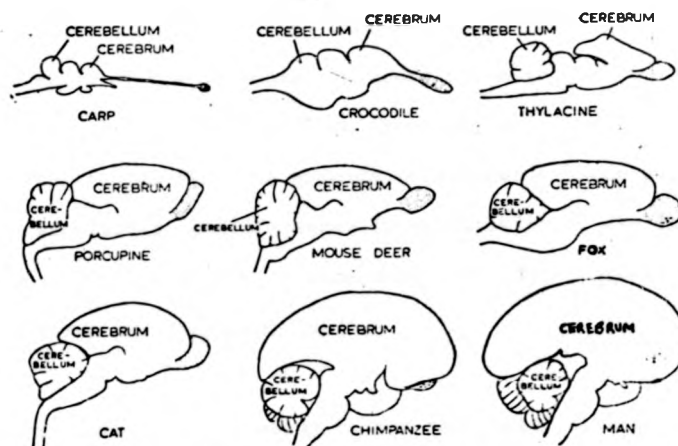


Fig. 1. Changes in relative size of the olfactory bulb with phylogenetic development of the brain.

(From Ottoson and Shepherd, 1967).

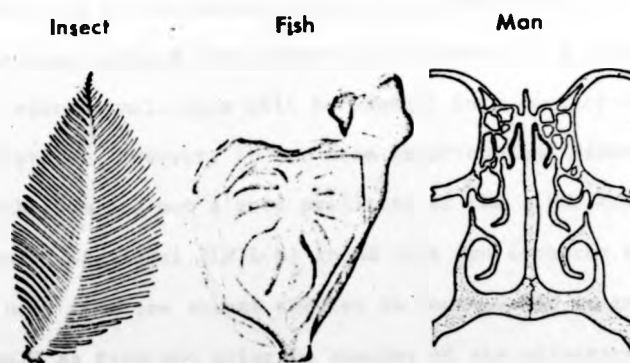


Fig. 2. The peripheral olfactory organ in insect (from Boeckh, 1962), fish (from Liermann, 1933) and man.

(From Ottoson and Shepherd, 1967).

and Shepherd, 1967). The general importance of olfaction, however, does not only depend on the relative part of the brain serving olfactory functions, but is also closely related to the functional characteristics of the peripheral sensory organ (Fig. 2). It is known from behavioural studies that there are wide variations in olfactory thresholds from one species to another. These changes have been attributed to differences in sensitivity of the individual receptors, the total number of receptors and their anatomical distribution (Ottoson and Shepherd, 1967).

A comparison of the anatomy of the peripheral olfactory sense organ in animals with different smell acuities shows that there is a direct relation between the area covered with sensory cells and the olfactory acuity. For instance, Allison reported that the area occupied by the olfactory mucosa in the nasal cavity is about $2 - 4 \text{ cm}^2$ for humans whilst the rabbit has 9.3 cm^2 ; Negus reported that a cat has 20.8 cm^2 (Graziadei, 1971 a). Dröscher (1971) said in his book that the olfactory area of the German sheepdog's nose measures 150 cm^2 . With a large area covered with sensory cells there is a greater probability that odorous molecules will be brought into contact with sensory receptors. However, it has been reported that dimensions of sensory epithelium are not a good predictor of the olfactory acuity of the animal. Graziadei (1971 b) found that the receptor cell density varies not only from one animal species to another, but in the same animal, as well as from one point to another of the olfactory epithelium. In insects, a very small area of the receptor membrane of the sensory

Table 1. Olfactory thresholds of man and worker honey bees (Kaissling, 1971)
(Threshold concentrations in molecules/cm³ air).

| Odour | Man | Author | Bee |
|-----------------------|---------------------|--------------------|---------------------|
| Propionic acid | $4.2 \cdot 10^{11}$ | V. SKRAMLIK (1948) | $4.3 \cdot 10^{11}$ |
| Butyric acid | $7.0 \cdot 10^8$ | V. SKRAMLIK (1948) | $1.1 \cdot 10^{11}$ |
| Valeric acid | $6.0 \cdot 10^{10}$ | V. SKRAMLIK (1948) | |
| iso-Valeric acid | $4.5 \cdot 10^{10}$ | SCHWARZ (1955) | $1.6 \cdot 10^{11}$ |
| Caproic acid | $2.0 \cdot 10^{11}$ | V. SKRAMLIK (1948) | $2.2 \cdot 10^{11}$ |
| Ethyl caproate | $1.3 \cdot 10^{11}$ | SCHWARZ (1955) | $3.8 \cdot 10^{11}$ |
| Ethyl caprylate | $3.7 \cdot 10^{10}$ | SCHWARZ (1955) | $5.4 \cdot 10^{10}$ |
| Ethyl pelargonate | $3.1 \cdot 10^{10}$ | SCHWARZ (1955) | $3.7 \cdot 10^{10}$ |
| Ethyl caprate | $4.2 \cdot 10^9$ | SCHWARZ (1955) | $5.0 \cdot 10^9$ |
| Ethyl undecylate | $1.4 \cdot 10^{10}$ | SCHWARZ (1955) | $1.8 \cdot 10^{10}$ |
| Methyl anthranilate | $2.6 \cdot 10^{10}$ | V. SKRAMLIK (1948) | $1.9 \cdot 10^9$ |
| Phenyl propyl alcohol | $6.5 \cdot 10^9$ | SCHWARZ (1955) | $2.2 \cdot 10^9$ |
| Nerol | $5.7 \cdot 10^9$ | SCHWARZ (1955) | $3.2 \cdot 10^9$ |
| Ionone-a | $3.1 \cdot 10^9$ | ZWAARDENAKER | $1.5 \cdot 10^{10}$ |
| Eugenol | $8.5 \cdot 10^{11}$ | Ohma | $2.0 \cdot 10^{10}$ |
| Citral | $4.0 \cdot 10^{11}$ | V. SKRAMLIK (1948) | $6.0 \cdot 10^{11}$ |

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cells is exposed to air, and it has been reported that a high sensitivity has been achieved by the low threshold of the individual organs (Table 1)(Kaissling, 1971).

1.2. FUNCTIONS OF OLFACTORY SYSTEM

a) Discrimination

The olfactory system is not only very sensitive, but it also has a great power of discrimination between odorous substances. An untrained person can classify about 2000 different odours, and a perfumer or trained chemist about five times that number (Döving, 1967). It is generally believed that the differentiation takes place at the level of the olfactory epithelium, and it was suggested that there are two possible mechanisms by which the olfactory mucosa could distinguish the odours:

- 1) by the existence of different types of receptors with selective sensitivity to different odorants;
- 2) by a spatial distribution of the odours in the olfactory epithelium (Adrian, 1953, 1956).

The first electrophysiological evidence for the existence of olfactory receptor types of different sensitivity was obtained by Ottoson who observed the receptor potential (EOG)* in frogs during selective olfactory adaptation (Ottoson, 1956). It was shown that the amplitude of the receptor potential decreased (for butanol) after the epithelium had been repetitively stimulated with butanol, whereas

* Electro-Olfacto-Gram

the amplitude of the receptor potential for amyl acetate did not decrease.

Physiological mechanisms underlying discrimination at the receptor level have also been studied with single unit recording techniques (Gesteland et al., 1963; Shibuya and Shibuya, 1963; Takagi and Omura, 1963; Gesteland et al., 1965; Shibuya and Tucker, 1967; Shibuya, 1969; Altner and Boeckh, 1967; O'Connell and Mozell, 1969; Mathews, 1972; Shibuya and Tonosaki, 1972; Daval et al., 1972). Using different stimuli, receptor types with different patterns of response were found.

b) Adaptation

It is a common experience that the perception of constant odour stimulation diminishes with time. Experimental results support the view that the olfactory organ slowly adapts both continuous and repetitive stimulation (Ottoson, 1956). It was found that repeated stimulations at short time intervals produced responses which after an initial decline remained almost constant in height during the subsequent stimulations, provided weak stimuli were used. Stuiver showed that recovery was quite rapidly completed at the unadapted side of the nose, but that the adapted side regained its original sensitivity only very slowly after an initial phase of rapid but incomplete recovery (Döving, 1967). The demonstration of the comparatively slow adaptation of the olfactory receptors was also found by Ottoson, who suggested that this type of response might imply that the phenomenon of olfactory fatigue has a central origin (Ottoson, 1963).

c) Olfactory Influence upon Behaviour and Body Function

The sense of smell is used by many animals to avoid enemies as well as to search for food and a mate. This sense is less critical for the survival of man. It may be used to detect spoiled foods and noxious gases, and for the appreciation of pleasant odours, such as perfumes, flowers and fine foods.

The influence of the sense of smell on the amount of food eaten was studied (Le Magnen, 1971). It was demonstrated that rats offered four different flavoured diets ate a regular amount of each diet, after an adaptation time of about one month. However, when a mixture of all four flavours was given, the amount consumed was greater than when they were given separately.

The sense of smell also plays an important role in the communication and reproduction amongst some forms of small animals. For instance, communication among insects occurs mainly by the transfer of chemical substances excreted by specific glands. These substances which are called pheromones act as stimulants of specific responses such as mating, feeding or alarm, as attractants, repellents or deterrents and as trail substances (Schneider, 1971).

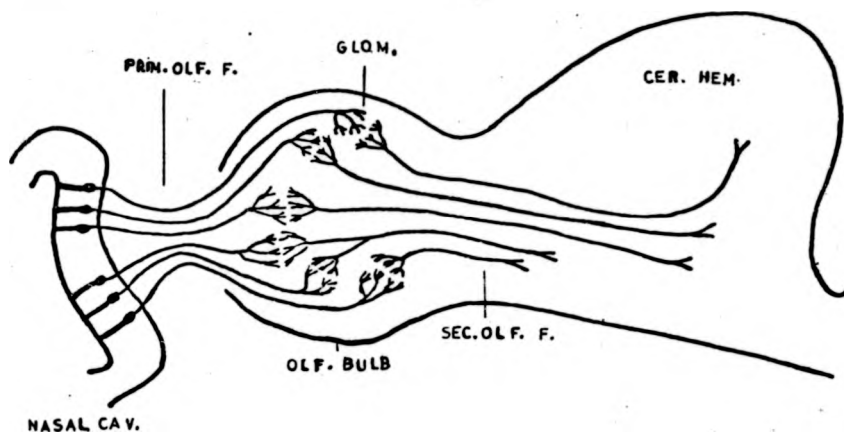


Fig.3. Scheme of the organization of the peripheral and central olfactory system in vertebrates. CER.HEM. : cerebral hemisphere; GLOM : glomerulus; NASAL CAV. : nasal cavity; OLF.BULB : olfactory bulb; PRIM.OLF.F.: primary olfactory fibers; SEC.OLF.F.: secondary olfactory fibers. (from Köster, 1971).

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1.3. MORPHOLOGY OF THE OLFACTORY SYSTEM

As is shown in Fig. 3 the olfactory system in vertebrates may roughly be divided into three parts: 1) a peripheral part represented by the Olfactory epithelium and primary olfactory fibres; 2) the olfactory bulb with secondary olfactory pathways; and 3) the cerebral hemispheres. Very few studies have been done on morphology and function of the central olfactory system in vertebrates. Only the first peripheral part will be described here because the work which has been done in this thesis is related to that, ^{the} olfactory mucosa.

Olfactory Mucosa

The vertebrate olfactory mucosa is that part of the lining membrane of the nasal cavities that contains olfactory receptor neurons. It is located in the upper posterior part of the nasal septum and the opposite region of the wall of the superior concha. It has been described that the mucosa is structurally different from the surrounding respiratory mucosa in many important respects,

- a) the presence of Bowman's gland,
- b) a characteristic yellow to brown colour,
- c) cilia not coordinately beating as opposed to the rhythmically beating cilia of the respiratory regions (Grazia~~x~~dei, 1971a)

The mucosa may be divided into two; the mucus layer and the olfactory epithelium. The former (covering the epithelium with the thickness of $10\mu - 40\mu$) is produced mostly by subepithelial Bowman's glands, whose ducts open onto the epithelial surface. The latter,

entral
al hemisphere;
BULB : olfactory
LF.F.: secondary

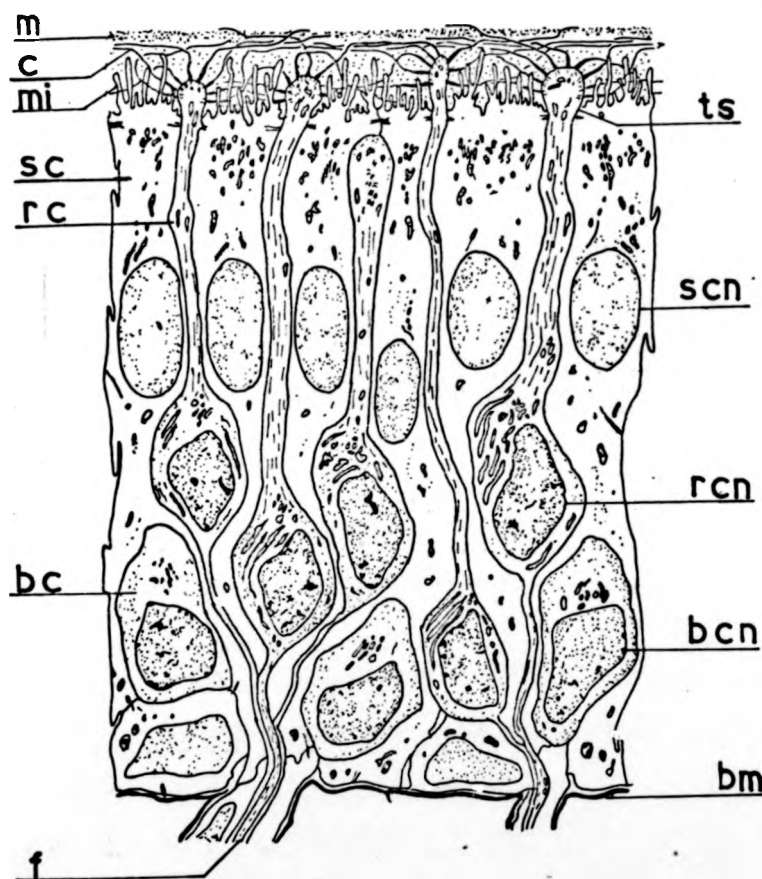


Fig.4. A half-schematic representation of the olfactory epithelium of vertebrates. m; mucus layer, c; cilia, mi; microvilli, sc; supporting cell, rc; receptor cell, bc; basal cell, ts; terminal swelling, scn; supporting cell nucleus, rcn; receptor cell nucleus, bcn; basal cell nucleus, bm; basal membrane, f; olfactory fila.

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as is shown in Fig. 4, consists of three types of cells: olfactory receptor cells, sustentacular or supporting cells, and basal cells. The receptor cells are actual nerve cells (bipolar neurons). From the peripheral part of the cell body there extends a thin process, the olfactory dendrite, that terminates at the outer limiting membrane with a rounded enlargement, the olfactory terminal swelling. A number of cilia protrude from the terminal swelling into the mucus. The proximal part of the cell body tapers down into a fine process, the axon, that continues as the olfactory nerve fibre and ultimately terminates in the olfactory bulb.

Histological sections showed that the olfactory epithelium is thicker than the surrounding respiratory epithelium. Its thickness, however, varied from one animal to the other ranging from 30 μ in moles to 150 - 200 μ in frogs and turtles (Graziadei, 1971b)

The olfactory neuron has a flask-like shape, its length is directly proportional to the thickness of the epithelium. The olfactory terminal swelling, ubiquitously provided with cilia, has an approximately diameter of 2 - 3 μ , the dendrite of 1 - 2 μ , the cell body of 5 - 8 μ . The axon is always unmyelinated and soon after its origin attains a diameter of 0.2 - 0.3 μ which is constant during its entire course to the olfactory bulb (Graziadei, 1971b)

The structure of cilia does not vary greatly from one species to another. They all have the typical 9 + 2 pattern of filaments,

well known in all cilia throughout the animal kingdom. Variations, however, have been found in their length and number. Okano et al. (1967) found some hundred cilia per receptor cell in dogs, while Graziadei (1966) found only a few in moles. Reese (1965) reported that the olfactory cilia of the frog were 200 μ long, whereas in lamprey they are only 5 - 6 μ long (Thornhill, 1967).

Supporting cells of the mucosa are columnar epithelial cells which lie between the receptor cells. They bear small numbers of irregular microvilli and have prominent oval nuclei situated basally.

1.4. THE OLFACTORY MUCUS AND PIGMENT

In all classes of vertebrates a layer of mucus separates the external environment from the surface of the olfactory receptors.

The composition of mucus is chemically only poorly known. Various enzymes, such as acid phosphatase, have been demonstrated histochemically at the olfactory surface and in Bowman's glands of mammals, and different mucosubstances have also been reported in various vertebrates, including frogs (Baradi and Bourne, 1953). Mucus in different parts of epithelia of the nose is known to have the following items: the pH of mucus is 7.0 or slightly acid, and the composition is over 95 per cent water. Salts and mucin (glycoprotein) are present in small amounts. Two other constituents of the mucus layer are muramidase, formerly known as lysozyme, and immunoglobulin A, or secretory immunoglobulin. It has been reported that these items might also be present in the

9

olfactory mucus (Abramson and Harker, 1973).

Recent findings indicated that the upper layers of mucus might be more mobile than the deeper ones; it is possible that water resorption by the supporting cells increases the concentration of non-diffusible substances near the epithelial surface (Bannister, 1974).

Smell is primarily a physico-chemical process; the odorous molecule must be in solution in order to be perceived. Therefore humidification is important in olfaction, and it was reported that olfactory epithelium is very sensitive to drying. Chronic forms of nasal dryness, such as atrophic rhinitis are frequently associated with anosmia, smell blindness (Abramson and Harker, 1973).

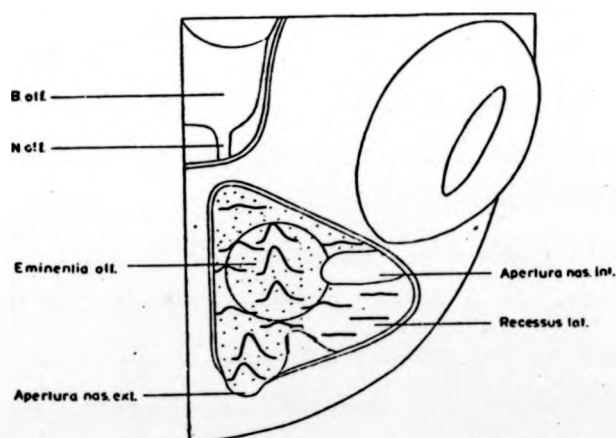
The functional significance of the pigment that gives the mucosa its characteristic yellow colour has been the subject of a great deal of speculation. Ottoson (1963) in his paper described the work of Ogle, who in 1870 suggested that the olfactory pigment might be associated with the absorption of some radiation from the odorous particles. The pigment would, according to this hypothesis, have a function similar to that of photochemical substances in the visual cells. It has also been assumed that there is a correlation between the degree of pigmentation and the acuity of olfaction. This idea appears to derive from statements that albino animals in general possess a poorly developed sense of smell (Moncrieff, 1951). Recently, data have been presented which seem to refute this hypothesis. When



From Ottoson (1956)

FIG. 5. RESPONSE OF THE OLFACTORY EPITHELIUM OF FROG TO STIMULATION WITH BUTANOL (EOG)

Upward deflection indicated negativity of the recording electrode. Vertical bar 1 mV. Time bar 2 sec.



From Ottoson (1956)

Fig. 6. DISTRIBUTION OF THE EOG IN THE NASAL CAVITY OF FROG. Dotted area indicates olfactory epithelium.

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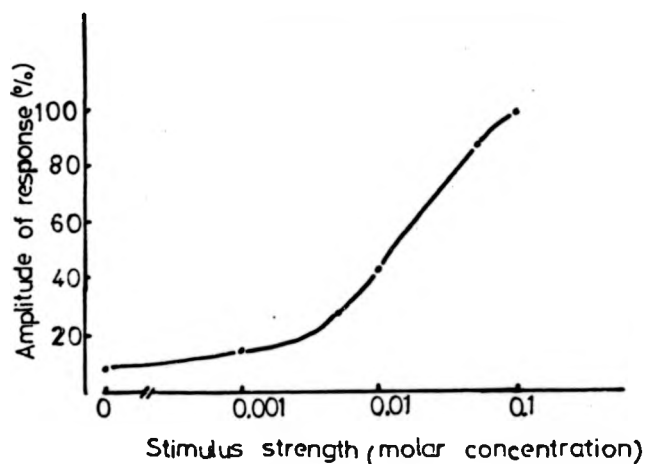
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albino rats were compared with pigmented littermates no significant differences were found in either the sensitivity to odours or the depth of pigmentation of the olfactory mucosa (Moulton, 1971). It was earlier reported that albino rats and rabbits have a normally pigmented olfactory mucosa that give normal responses to odours when tested by electrophysiological methods (Jackson, 1960). It was suggested that the olfactory pigment was composed of phospholipids, probably lecithins, plus the products of auto-oxidation of these phospholipids. The latter contributed most, or all, of the yellow colour of the olfactory mucosa (Jackson, 1960).

It has recently been suggested that ascorbic acid does not play a direct role in the transduction mechanism; under certain conditions it could inhibit the auto-oxidation of unsaturated phospholipids and might inhibit the presence of a lipofuscin pigment in the olfactory mucosa (Dodd and Cash, 1973).

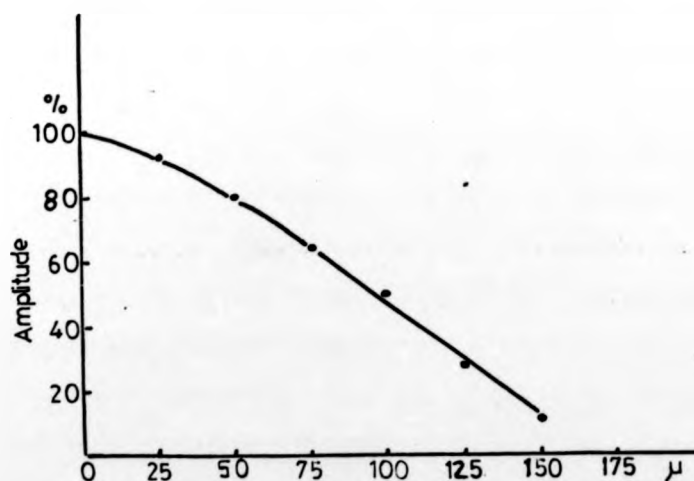
1.5. THE ELECTRICAL ACTIVITY OF THE OLFACTORY MUCOSA

When the olfactory epithelium is stimulated with a short blow (one second) of odorous air, the mucosa responds with a slow change in electric potential. Shortly after the molecules hit the epithelium (0.3 sec.) there is a swift negative phase which reaches a maximum and returns to the original baseline several seconds after the end of stimulation (Fig. 5). This monophasic negative change in potential of the receptor epithelium was first reported in 1937 by Hosoya and Yoshida (1937), but the first detailed study came in 1956



From Ottoson (1956)

Fig. 7 RELATIONSHIP BETWEEN STIMULUS STRENGTH (BUTANOL) AND AMPLITUDE OF THE EOG



From Ottoson (1956)

Fig. 8 THE AMPLITUDE OF THE EOG WITH A CONSTANT ODOR STIMULUS, RECORDED WITH A MICROELECTRODE AT DIFFERENT DEPTHS IN THE RECEPTOR LAYER

from Ottoson (1956) with one electrode in the eminence of the brain. This monopolar recording (Fig. 6) showed no response to the stimulus. It is a mass recording of the amplitude of the EOG. If a microelectrode is used, the usual 100% response is obtained. Different forms, and the response is a new one. The EOG is recorded from the cilia.

Since the EOG was established in 1959 (Fig. 9) with great detail (Ottoson, 1961).

from Ottoson (1956). He worked with ^{the} decapitated head of a frog, with one electrode touching the surface of the exposed olfactory eminence and the reference electrode beneath the head. He called this monophasic change the electro-olfactogram (EOG) (Fig. 5). Fig. 6 shows the distribution of the EOG in the nasal cavity of the frog. The EOG was obtained from the sensory epithelium, whereas no response was elicited from the respiratory epithelium. The EOG is a mass response from all the receptors of the epithelium, the amplitude of which is dependent upon the stimulus strength (Fig. 7). If a microelectrode about 0.5μ in diameter was used, instead of the usual $100 - 200\mu$, the EOG was found to be greatest at the surface and to fall off steadily with depth, to zero at about 150μ (Fig. 8). Different stimuli produced responses of slightly different wave forms, and after a series of conditioning puffs with one odour, the response to the same odour diminished more than the response to a new one. As a result of these experiments, Ottoson proposed that the EOG was a generator potential, originating in the olfactory cilia.

Since Ottoson's original work with which the negative "on" EOG was established, four other types of EOG responses have been found. In 1959 Takagi and Shibuya (1959) reported a negative "off" wave (Fig. 9). Takagi and his co-workers have studied this potential in great detail, including its underlying ionic mechanisms (Takagi et al., 1969).

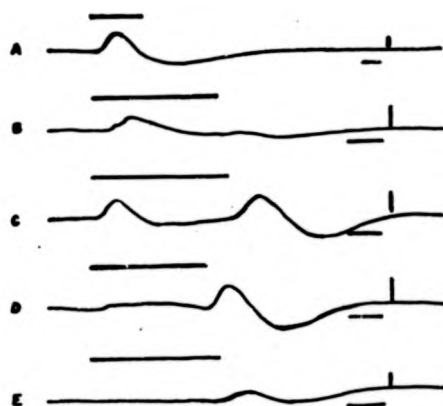


Fig. 9 On- and off-responses. (A) On-response. (B), (C), and (D) Various types of on-off-responses. The on-response is bigger than the off-response in (B) and vice versa in (D). (E) Off-response. The horizontal lines above the potentials on the left indicate the durations of olfactory stimulation. The vertical and horizontal bars on the right show 50 μ V and 1 sec, respectively. (From Takagi & Shibuya, 1959.)

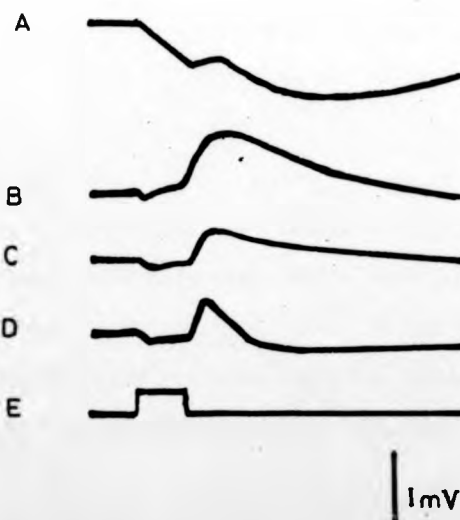


Fig. 10 Positive EOGs of various types. (A) Typical EOG elicited by chloroform vapor. This shape is always observed in the ceiling olfactory epithelium of the nasal cavity. (B) Small on-potential is followed by a large negative off-potential. This is often followed by a small positive afterpotential. (C) and (D) Intermediate shapes between A and B. The EOGs in B, C, and D are most often observed in the olfactory eminentia. Time of stimulation is indicated at bottom right. (From Takagi, Aoki, Masao, & Yajima, 1969.)

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Positive EOG potentials have also been discovered. Takagi et al, (1960) first recorded the positive "on" potential in frogs, and Shibuya (1960) recorded a positive "off" potential in fish (Fig. 10). MacLeod has reported a positive afterpotential type of EOG in rabbits (MacLeod, 1965).

Recently Ottoson (1971) defined the EOG as the 'monophasic negative potential' evoked by odours in the sensory region of the nasal mucosa. However, in the retina the term 'ERG' is used to define the complicated potentials with the positive and negative components all of which are elicited in the retina by light illumination. Since it is certain that the potential elicited in the olfactory epithelium by odours contains not only the negative components but also the positive components, it has recently been proposed that the word 'EOG' should be used in a wide sense, namely for all kinds of potentials which are evoked by odours in the olfactory epithelium (Okano and Takagi, 1974).

In order to clarify the origin of these EOGs, Takagi and Yajima sectioned the olfactory nerve and studied both the degeneration of the olfactory epithelium and the change of the EOGs (Okano and Takagi, 1974). They found that the olfactory cells degenerated and disappeared in proportion to the decrease in amplitude and disappearance of the negative EOGs, but that the supporting and basal cells survived together with the long-lasting positive potential. Consequently, the origin of the negative EOGs was attributed to the olfactory cell.

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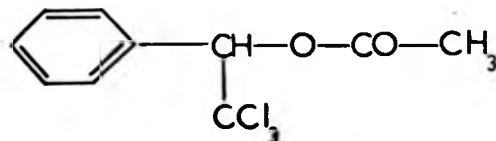
Evidence that this is so has been provided by Minor (1971) who has compared the EOG in frogs with the "global" olfactory receptor generator potential recorded from the olfactory nerve. The olfactory nerve had been treated with procaine to block spike activity. The two records were closely parallel and Minor concluded that EOGs originate in the receptors.

When chloroform and some other odours are applied onto the olfactory epithelium of the bullfrog, the long-lasting positive potential appears and continues over several hundred m sec. (Takagi et al., 1966; 1968). The origin of the long-lasting positive potential remained unsolved until recently. In the recent studies, while the long-lasting positive potential continued, the epithelium was instantaneously fixed. An electron-microscopic study disclosed remarkable secretion in the supporting cells, which, in contrast, was not found in the epithelia producing the negative slow potentials in response to amyl acetate and other odours (Okano and Takagi, 1974). Secretion of the supporting cells was also observed in the degenerating epithelia in which the olfactory mucosa had been sectioned previously. From these studies it was concluded that the secretion of the supporting cells is the most potent, possibly , origin of the long-lasting positive potential.

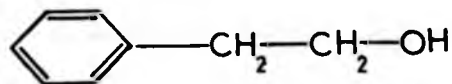
1.6. ODOURS, RECEPTORS AND ODOUR-RECEPTOR INTERACTIONS

Modern theories agree that a substance must be volatile in order to serve as an olfactory stimulus. This requirement has reduced the

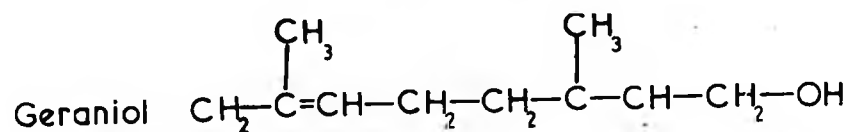
Rosetone



Phenylethanol



Geraniol



Pelargol

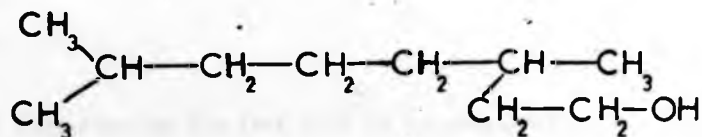


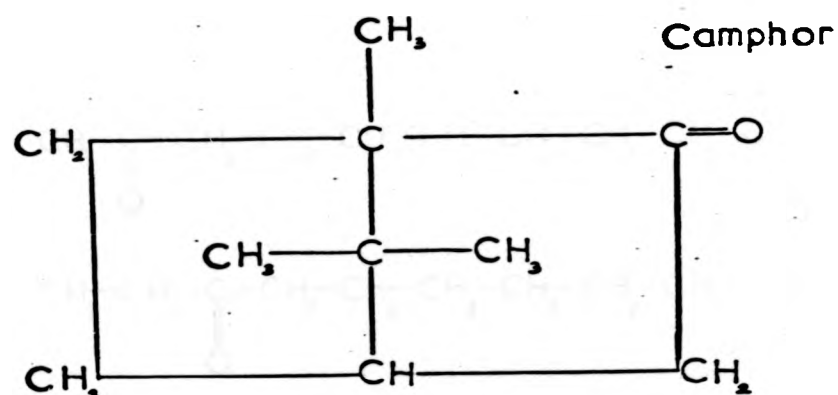
Fig. 11. Compounds with a roselike smell.
(from Vinnikov, 1974)

number of possible odorants drastically since volatile substances have, in general, a molecular weight of less than 300 (Døving, 1974). Solubility in water as well as lipoids appears to be a common property of olfactory stimuli, and most theories include this factor in their list of important characteristics.

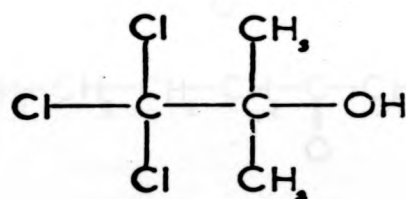
The chemical properties of compounds are known to be associated with their structure; however, molecules of different structure may have a similar smell, and vice versa. For instance substances with quite different structures (Fig. 11) have a "roselike" smell. Fig. 12 shows another example of compounds with a camphor smell (Wright, 1964, 1966). An example can also be given for odorants which have molecules of similar structures but different odours. In 1929 Braun, Kröper and Veinhaus made a series of compounds (Fig. 13); all these compounds have an odour resembling that of rue, but with the shifting of the carbonyl group ($-CO-$) towards the middle of the chain the rue odour becomes weaker and gradually changes into a fruity odour (Vinnikov, 1974).

Amoore's theory was supported by the fact that it is sometimes possible to predict the odour of a compound from the shape of its molecule. For example, the probable odour of molecules of the three compounds (Fig. 14) was predicted by Rubin, Apotheker, Lutmer, 1962. (Vinnikov, 1974).

The molecule of the first compound is a rather compact sphere and fits in the best possible manner into the "camphoraceous" site.



Chloroethane



Ethyl-tret-butylether

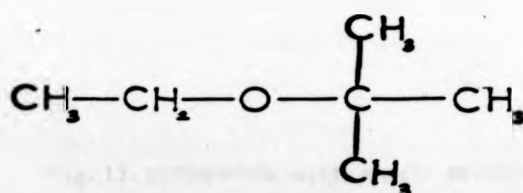


Fig.12. Compounds with a camphor smell.
(from Vinnikov, 1974)

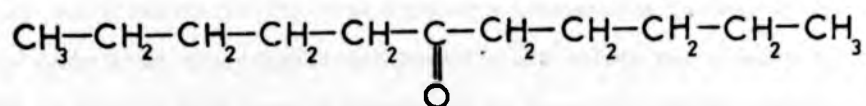
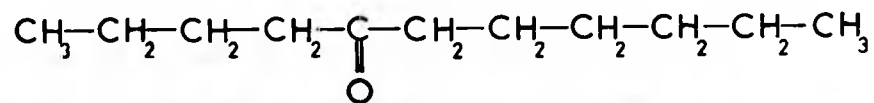
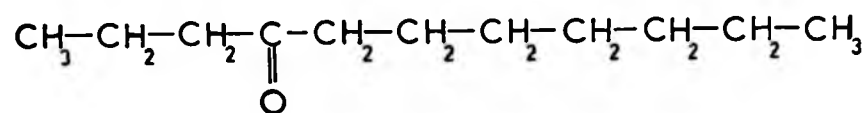
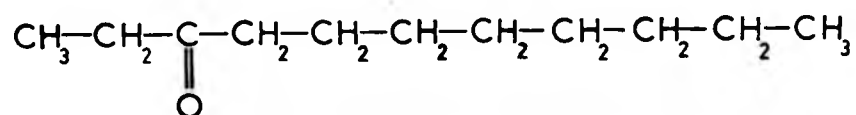
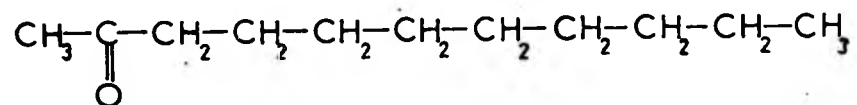


Fig. 13. compounds with similar structures but different odours.
(from Vinnikov, 1974)

The molecule of the third compound has a very long "tail" and, therefore, fits a "floral" site best. The second compound corresponds, in molecular shape and size, to both types of site mentioned above and hence would be expected to yield a combination of both odours. These predictions were confirmed in practice (Johnston and Sandoval, 1962). In addition to the compounds in the Fig. 14, several analogous compounds were synthesised and found to have the predicted quality and intensity of odour (Johnston & Sandoval, 1962).

There has been some controversy on the question of the location of olfactory receptor sites in recent years. The fact that the cilia present a considerable surface area very close to the surface of the mucus has supported the idea that they carry the receptors. Ottoson was the main supporter of this argument and has pointed to the short latency of the EOG and the way it declines with depth in the epithelium (Ottoson, 1970). Atema has recently proposed that the ciliary microtubules are the true olfactory transducers (Atema, 1973). On the other hand, when Tucker stripped the cilia from the olfactory mucosa of turtles with aqueous detergents he found the neural response to odours were only partly affected (Tucker, 1967).

It has been assumed that there are molecular receptors, or active sites for a receptor molecule, in the receptor membrane which interact with the stimulus molecule (Gesteland et al., 1965; O'Connel and Mozell, 1969; Beets, 1971; Getchell and Gesteland, 1972; Polak, 1973).

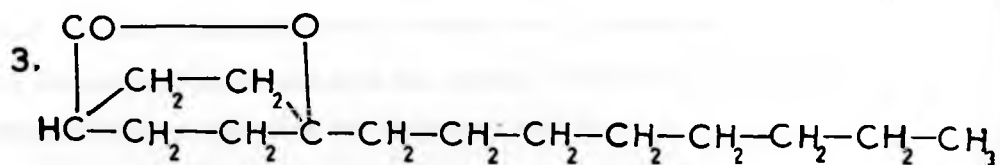
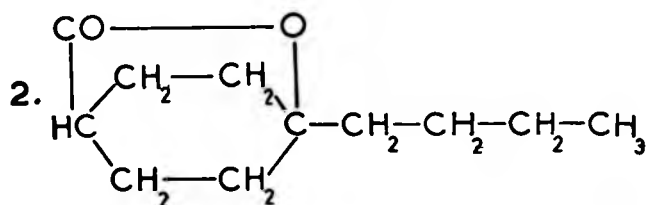
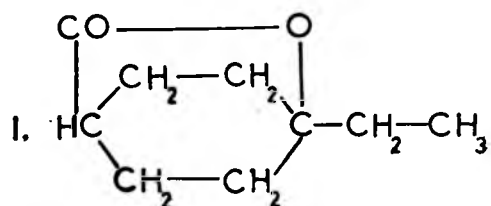


Fig.14. Compounds with predicted smells(see text)
(from Vinnikov 1974)

They implied that the receptor is most likely to be proteinaceous since proteins are the only macromolecules known to possess a wide enough variety of shape to account for the possibilities of odour discrimination.

The importance of proteins rather than lipids in the processes of olfaction is supported by the findings of a number of workers. The first electrophysiological evidence indicated that individual receptor cells behave in different ways when stimulated by a single odour (Mozell, 1964, 1971; Gesteland *et al.*, 1965). Holley (1974) suggested that this was caused by a variation in the ratio of specific receptor sites on each sensory cell pointing to the likelihood of membrane proteins being involved.

Ash (1969) observed spectrophotometrically in preparation of rabbit olfactory epithelium that changes occur in absorbance at 267 nm when the odorant interacted with the tissue, pointing to an interaction between stimulant molecules and olfactory proteins.

Getchell and Gesteland (1972) found that the group-specific protein reagent, NEM irreversibly blocks the electrical response of the olfactory receptors of the frog to odour stimuli.

1.7. THEORIES ON THE OLFACTORY TRANSDUCTION MECHANISM

Previous studies of olfaction have attempted to link an odour type with one or more physical characteristics of compounds, and over the

last century several theories have been proposed correlating odour with feature of molecular structure. Now the search is for a general structure-activity relationship. Correlations of chemical structure with biological activity has just been reviewed in the ECRO* symposium (Ed. G. Benz, 1976).

Most of the old theories of olfaction regard the initiation of the nervous discharge as the result of chemical reactions between the odours and the olfactory receptors. Ruzicka suggested that activation of the olfactory receptors occurs as the result of reactions between what he called osmoreceptors in the olfactory membrane and certain functional groups such as -OH, -CHO, -CO, -COOR. -CH, NO₂ and N₃ in the odorant molecule. He then modified this theory and proposed that the odoriferous groups trigger enzymatic reactions which are the direct cause of the initiation of the nervous impulse (Ottoson, 1963).

Moncrieff (1951) proposed a theory of olfaction based on the ability of the odorant to fit into sites of specific shapes in the olfactory membrane. Amoore starting from the assumption made by Moncrieff (1951) that the odour of a compound depends on the shape of its molecules and how accurately this molecule can be "inscribed" into a "specific site" on the surface of a receptor cell, formulated his stereochemical theory of olfaction (Amoore, 1962a, 1962b). According to his stereochemical theory, there is a limited number, originally 7 (Amoore, 1952), of receptor site types characterised by their shapes

* European Chemoreception Research Organization

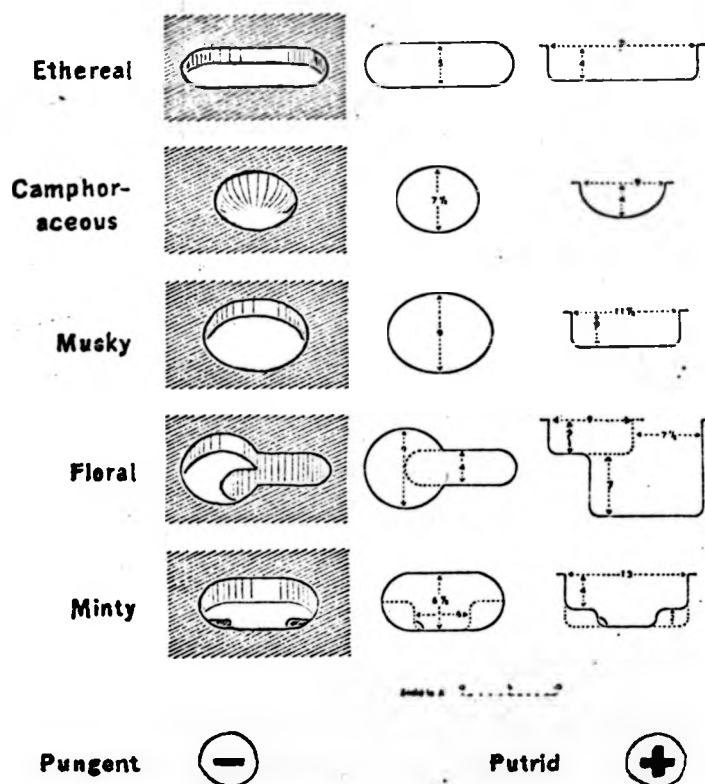


Fig.15. The human olfactory receptor sites, corresponding with the seven primary odors. On the left are oblique perspective drawings, in the center the plans of the sites seen from above, and on the right the elevations of the sites seen from the front. The dimensions are in A units.

from Amoore (1967)

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(Fig. 15). It postulates that compounds of similar odour must also have a similar molecular geometry. If the conformation of a molecule of the compound corresponds specifically to only one site on the surface of the receptor cell, such a compound will possess a "primary" odour. For example, the musk receptor site would be an oval pan-shaped depression in the membrane 9\AA wide, $11\frac{1}{2}\text{\AA}$ long and at least $3\frac{1}{2}\text{\AA}$ deep. Other receptor sites described by this kind of model were champeoraceous, floral, minty and ethereal. Pungent and putrid odours do not fit into this scheme. The molecules responsible for these odours have indefinite shape and size. The pungent odour group is associated with compounds whose molecules have electrophilic functional groups (and therefore, their receptor sites should contain nucleophilic functional groups) while putrid odours are emitted by molecules having nucleophilic functional groups. Complex groups (such as almond, lemon, aniseed, etc.) occupy two or more of the receptor sites for the primary odorants. The original list of seven primaries has been expanded considerably (Amoore, 1967). Amoore has recently considered that specific anosmias are caused by the inherited absence of the appropriate receptor protein for a given primary odour (Amoore, 1970). A specific anosmia is the inability to detect or recognise the odour of a particular compound though other odours are perceived normally.

A profile-functional concept has been developed by Beets (Beets, 1957), who has made an extensive study of the relationship between molecular structure and odour quality (Beets, 1971). He goes a long way with Amoore on the concept of the receptor site and the importance

of molecular shape, but he has stressed the role that functional groups can have in determining the preferred orientation of the molecule at the receptor site.

Wright (1957) suggested that the mechanism of olfaction is analogous to that of vision, involving the reception by olfactory pigments. According to Wright's theory, for odorant molecules to cause stimulation of olfactory cells, they should not only come into intimate contact with such cells but must also act at the same time on olfactory pigment present in these cells. Molecules of olfactory pigment absorb low-frequency vibrations emitted by molecules of odorants; this is followed by a series of energy conversions, culminating in nerve impulses transmitted to the central nervous system. Wright (1966, 1967) and Wright and Robson (1969) studied the far infrared spectra of extensive series of musks and other odorants. The spectra of all musks seem to be related by the presence of three frequencies near 100, 160 and 260 cm^{-1} . The spectra of odorants with bitter almond odour, such as benzaldehyde and nitrobenzene, show bands near 175, 225 and 345 cm^{-1} , while the cuminic odour of higher homologs is associated near 175, 265 and 310 cm^{-1} . This theory cannot account for the fact that some optical isomers have the same absorption spectra but quite different odours (Young *et al.*, 1948). Also the recent substantiation (Friedman and Miller, 1971) of the fact that the enantiomers R-(-)-carvone and S-(+)-carvone have distinctly different odours (spearmint and caraway respectively) argues against vibrational theories because enantiomers have the same vibrational frequencies.

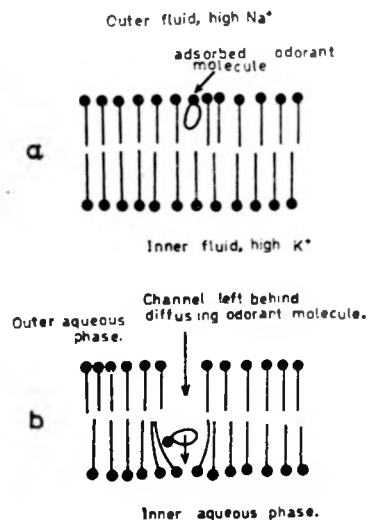


FIG. 16. In (a) is shown the supposed structure of the membrane constituting the cell wall, at the sensitive end of the olfactory cell. The odorant molecule is shown adsorbed and oriented among the lipid molecules constituting the boundary of the membrane. In (b) the odorant molecule is shown diffusing through and out of the membrane. It is assumed here that the membrane heals relatively slowly, so that an aqueous channel is left behind the diffusing odorant molecule. Through this channel Na^+ and K^+ ions are postulated to exchange. (from Davies, 1959)

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There are some theories that are specifically designed to explain the receptor process itself and have little or nothing to say about the properties of the stimulus. Perhaps the best known of these is Davies' puncturing theory (Davies, 1954, 1959, 1965). It suggests that odour molecules are adsorbed into the lipid membrane of the receptor cell. When it is then desorbed (i.e. removed), the molecule is thought to leave a puncture in the membrane that remains open for a fraction of a second until the cell can repair the damage. During this time, an exchange of ions occurs (potassium flows out of the hole, sodium into it), and this initiates a propagated membrane disturbance in accordance with the general neurophysiological model (Fig. 16). Davies suggested that odour quality depends on the ratio between the healing of the membrane to that of diffusion of the odorant molecules through it (1965), which in turn depends on the desorption rate and geometry (principally area of cross-section) of the molecule. However, Cherry *et al.*, (1970) have shown that no correlation exists between resistance changes induced in lipid bilayers by odorants and the olfactory threshold of the odorants. For example, the ionones, which have very low olfactory thresholds yet have no significant effect on the bilayer resistance.

The enzyme theories (Kistiakowsky, 1950) suggested that odour molecules somehow inhibit one or more enzymes that are essential to normal cellular reactions. Such a disturbance could conceivably produce a momentary breakdown of the resting membrane potential and give rise to action potentials. Baradi and Bourne (1951a, 1951b,

1953) have tested the enzyme theory experimentally by measuring the concentration of enzymes localised in the olfactory mucosa after substances with different odours had been added to the substrate solution. They found a certain degree of specificity insofar as that the enzymes were inhibited differentially by different substances. One evidence against enzymatic theories is that the distal regions of the receptor cells, where the initial interaction between odorant and receptor probably takes place, are relatively lacking in enzymes (Herberhold, 1968). Furthermore it has been found that treatment of the olfactory mucosa with cholinesterase inhibitors such as neostigmine, and eserine does not block the olfactory response (Ottoson, 1963). In addition, the effects of ouabain, a specific inhibitor of the enzyme $(\text{Na}^+ - \text{K}^+) - \text{ATPase}$, on the EOG amplitude developed much more slowly (with n-butyl alcohol as the stimulus, the time required for 50% reduction in the amplitude of the EOG 25 minutes) than those caused by the group specific reagents, and they were not accompanied by changes in the EOG waveform like those seen with the protein reagents. It was therefore concluded that the inhibition of the ouabain-sensitive ATPase was not the cause of the effects produced by the group specific reagents.

1.8. AIMS OF THIS THESIS

It has become evident from the survey of the literature that the study of olfaction absorbs the activity of research workers from a diverse range of disciplines including physiology, organic chemistry, anatomy, zoology, psychology and perfumery. The bulk of the studies

on olfaction have been electrophysiological. Biochemistry has been used in a few studies (Koch and Norring, 1969; Dodd, 1970; Koyama et al., 1971 and Menevşe et al., 1974) as a discipline in elucidating the transduction mechanism involved in olfaction. As Amoore (1974) has stated, "the sense of smell involves the interaction of a biological detector, the nose, with a chemical stimulus, the odour." Hence the most logical methods to attack the problem would appear to be those of biochemistry. However, it has become clear (Menevşe, 1973 and the second chapter of this thesis) that the application of biochemistry to study the olfactory mechanism at the molecular level is insufficient. To study the binding process between the odour and the receptor, which biochemistry does, it is essential to have an isolated and characterised biological system i.e. olfactory receptor. Because of the complexity of these studies in the heterogenous system like olfaction, the use of a combined chemical modification and electrophysiological technique is unavoidable.

It is presumed that the initial event in olfaction is the interaction of the odorant with the receptor sites on the neuronal membranes leading to a production of a receptor potential (Dodd, 1974).

In view of this the aims of this work were as follows:-

- 1) to attempt to isolate and characterise membranes from olfactory primary neurons,
- 2) to obtain an insight into the mechanism of the olfactory coding by chemical modification methods,
- 3) to elucidate the nature of the transduction step in olfactory stimulation.

CHAPTER 2

CHARACTERISATION OF OLFACTORY MEMBRANES

2.1 INTRODUCTION

It is known that cell membranes are not merely diffusion barriers, but are rather the location of many of the cell's major biochemical activities. For example, some important metabolic processes such as electron transport and oxidative phosphorylation, and other biochemical reactions are often associated with proteins located in membranes. In this respect, the plasma membrane surrounding living cells not only plays a role in intracellular metabolism, but also mediates interactions between the cell and its external environment. For instance, the plasma membrane is involved in cell secretion and recognition, excitation and impulse conduction, reception of hormones, chemotactic processes etc. It is likely that the transduction event in olfaction involves plasma membranes.

Subcellular fraction techniques, when applied to nervous tissue, provide methods for studying the location of transmitters and the enzymes that synthesise and degrade them, and thus the organisation of the synapse at the molecular level. Whittaker (1965, 1966) reviewed such studies used in the investigation of brain function. De Robertis (1967) presented a review dealing with work on the separation and isolation of nerve endings and of synaptic membranes in crude mitochondrial preparations. His research group (Arnaiz *et al.*, 1967) described the use of membrane bound enzymes as biochemical markers for

isolated synaptic membranes morphologically characterised by electron microscopy.

The isolation and characterisation of plasma membranes from different tissues has been reviewed (Solyom and Trams, 1972; Wallach and Lin, 1973; De Pierre and Karnovsky, 1973). The $\text{Na}^+ - \text{K}^+$ activated ATPase has been used as the main marker enzyme in the characterisation step of plasma membranes in different tissues.

Koch (1969) initiated the biochemical study in olfaction by way of adopting subcellular fraction techniques, and subsequently he used the $\text{Na}^+ - \text{K}^+$ activated ATPase as the main criteria in detecting the plasma membrane fragments from the rabbit's olfactory tissue. Dodd (1970) has also published a report on enzyme activity in homogenate fractions from olfactory tissue. His results showed the presence of ATPase activity in subcellular fractions from olfactory tissue homogenates from a number of different mammals. An attempt was also made by Koyama et al., (1971) to isolate the plasma membranes from the bovine olfactory epithelium using the activity of the $\text{Na}^+ - \text{K}^+$ ATPase as a marker. I also attempted to isolate plasma membrane fragments from the cow olfactory mucosa (Menevşe et al., 1974). Two methods were applied: morphological characterisation using electron microscopy and the determination of the enzyme profile.

The aim of this chapter was to provide a further study on the membranes of olfactory mucosa.

2.2 MATERIALS AND METHODS

All chemicals were of an analytical reagent grade from either Sigma Chemical Co., Ltd. or British Drug Houses. Solutions of chemicals were prepared with double distilled water.

All animals used were free from rhinitic infections. The heads of cows, calves and sheep were obtained from the local slaughterhouse. The rabbits and rats used were from the University's animal house.

The olfactory epithelium from the turbinates of both right and left sides was taken out immediately and cut into small blocks (1mm^3). The tissue was fixed in a solution containing 2% glutaraldehyde in 0.1M phosphate buffer pH 7.4 for 24 hours. The tissue blocks were then post fixed for 2 hours in 1% osmium tetroxide buffered at pH 7.4 with 0.1M phosphate buffer. The tissue blocks were then rinsed with the same buffer and dehydrated in a graded series of ethyl alcohol solutions, followed by propylene oxide and embedded in araldite. Thin sections obtained on a Reichert (Austria) ultramicrotome were stained with 2% uranyl acetate for 30 minutes and 1% lead citrate for 10 minutes. The thin sections were examined in an AEI Corinth 275 electron microscope.

Methods

Two different methods were employed for the isolation of the cilia from sheep olfactory mucosa. The methods used in this work were previously described for the isolation of rod outer segments from frog retina, (Bitensky et al., 1971); Method I, and for the isolation of cilia from the protozoan *Tetrahymena* (Watson and Hopkins, 1962); Method II.

Both methods involve the removal of the yellowish endoturbinates, which have olfactory epithelium, to be put into the solution concerned.

Method I The olfactory endoturbinates were collected in an ice-cold 47% sucrose solution (2 - 3 ml. per endoturbinate) and agitated vigorously for 1 minute on a Vortex mechanical vibrator. The endoturbinates were then removed from the solution and the solution was centrifuged at 1000Xg for 10 minutes at 4°C in a Spinco SW 34 rotor. The sediment was collected in 0.1M phosphate buffer pH 7.4 and named as G₁. The supernatant was then centrifuged at 2000Xg for 30 minutes and the sediment was collected in the same buffer and named as G₂. These two sediments, G₁ and G₂, were examined by electron microscopy after being either negatively stained with 1% uranyl acetate or fixed, dehydrated, embedded and thinly sectioned.

Method II The olfactory endoturbinates were collected in 0.025M sodium acetate buffer pH7.0 (2 ml per endoturbinate), and gently shaken

for 5 minutes (Step B). Endoturbinates were then placed into a solution which consisted of 12% (V/v) ethyl alcohol in 0.025M sodium acetate containing 0.1% EDTA (Step B₁). 2ml of 1M CaCl₂ was quickly introduced to the solution (100ml) containing the endoturbinates. The solution was gently shaken for 3 minutes. The endoturbinates were removed and the suspension was centrifuged at 1000Xg for 10 minutes (Step B₂). The supernatant of this centrifugation was removed by aspiration. The second pellet was obtained by centrifuging at 15000Xg for 20 minutes (Step B₃); small portions of every step of this method were subjected to examination in the electron microscope.

2.3 RESULTS AND DISCUSSION

The dendritic part of the primary neurons in the olfactory mucosa of several animal species has been examined. The results of electron microscopic studies are generally in agreement in the animals which were used, for example: cow, calf, sheep, rat and rabbit. The portion of the dendrite at, and beyond the epithelial surface, can be divided into two parts, namely the neck and the olfactory terminal swelling. The terminal swelling is usually columnar in shape and is immersed in the many bushy microvilli of neighbouring supporting cells (Figure 17).

The neck, i.e. the region of junction between the terminal swelling and the adjacent subsurface part of the cell, is somewhat smaller in diameter than the remainder of the dendrite (Figure 18).

The cilia protrude from all parts of the terminal swelling at different angles to their surface (Figure 19). A thick differentially oriented meshwork of cilia was thus formed among the protruding terminal swellings and the microvilli of the supporting cells (Figure 20).

The general internal structure of the ciliary shaft was found to be the same in the olfactory mucosa of all the animals studied. The length of cilia in olfactory preparations from different animals were found to be 10μ , although they are undoubtedly much longer, since the



Fig. 17. Terminal swelling (TS) of a sheep olfactory receptor cell.
C; cilium, Mi; microvilli, N; neck of the dendrite.



Fig. 18. Section of upper portion of cow olfactory epithelium.

RC; receptor cell, SC; supporting cell, TS; terminal swelling
C; cilium, Mi; microvilli.

entire longitudinal sections could not be seen in thin sections. Olfactory cilia, in animals used, measured between 0.25 and 0.3 μ in diameter at the base, and became gradually thinner towards the distal end. Although the number of cilia which derived from an olfactory terminal swelling was not ascertained, up to eighteen were counted in both cross and longitudinal sections in the calf mucosa. It can be estimated that the terminal swelling may contain more than that number, possibly 20 - 30 cilia.

Microtubules and oval vesicles were found in the terminal swelling of sheep and calf olfactory neurons. Mitochondria were observed only infrequently in the olfactory terminal swellings of all animals studied.

The part of the dendrite between the nucleus and the neck was found to contain numerous mitochondria.

Similar observations have been found in the olfactory mucosa of the frog (Graziadei and Gagne, 1973), mouse (Frisch, 1964), man (Naessen, 1971), and dog (Okana, Weber, Frommes, 1967). However, the length and the number of cilia varied from one specimen to another in vertebrates. Okana et al., (1967) estimated that a total of 100 - 150 cilia are present on each receptor cell of the dog. In the rabbit olfactory epithelium, the number of cilia were estimated to be between 30 - 60 (Mulvaney and Heist, 1970).

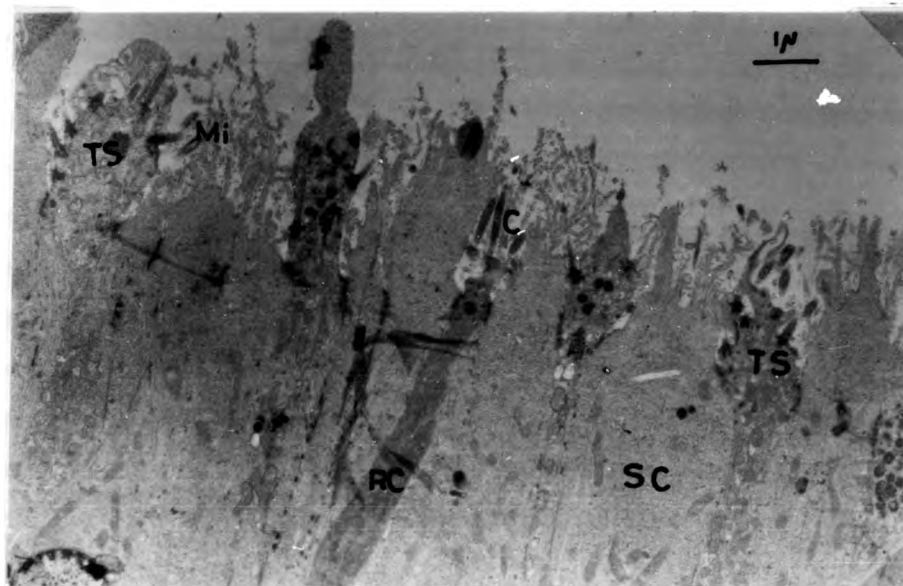


Fig. 19. Longitudinal section of a calf olfactory epithelium. TS; terminal swelling, Mi; microvilli, RC; receptor cell, C; cilium SC; supporting cell.



Fig. 20. Apex of rat olfactory terminal swellings (TS) cut parallel to the mucosal surface.

After having examined the structure of olfactory terminal swellings and cilia in several animal species, it has subsequently become clear that these elements of the primary neurons could be ideal structures for the characterisation of the receptor cell plasma membranes. However, as has been reported (Menco *et al.*, 1974; Menevse, 1975), during isolation procedures deciliation took place and it then became very difficult to distinguish the membrane vesicles of the sensory membranes from the membrane vesicles of the supporting and even basal cells. Since the olfactory mucosa with its different cell types represents a typical example of heterogenous tissue an enzyme profile analysis was also of limited use in determining the purity of a receptor cell plasma membrane fraction. The enzyme analysis was mainly of use in confirming both the presence of plasma membranes which are plasma-membrane-bound enzymes, such as $(\text{Na}^+ - \text{K}^+) - \text{ATPase}$ and adenylate cyclase, and the absence of intracellular membranes, all of which have enzymes which are not found in plasma membranes.

The isolation methods which were used are based on the procedures devised for the preparation of post-synaptic membranes of the brain (Whittaker, 1965). The method involves the following: the yellowish olfactory epithelia is first scraped off; homogenisation of the tissue is followed by discontinuous sucrose density gradient centrifugation and washing procedures. Several sub-fractions were obtained and assayed for the enzymes. As is seen from Table 2, the highest $(\text{Na}^+ - \text{K}^+) - \text{ATPase}$ was found in sub-fraction B. This fraction was fractionated further into five ($\text{B}_1 - \text{B}_5$) by discontinuous sucrose gradient centrifugation. Fractions B_2 and B_3 showed high $(\text{Na}^+ - \text{K}^+) -$

| Cell fraction | Total ATPase | Mg ⁺⁺ ATPase | Na ⁺ -K ⁺ , ATPase | % Na ⁺ -K ⁺ ATPase |
|---------------|--------------|-------------------------|--|--|
| WH | 2.88 | 1.84 | 1.04 | 36 |
| A | 2.48 | 1.85 | 0.13 | 25 |
| B | 7.40 | 4.80 | 2.60 | 35 |
| S | 3.12 | 2.69 | 0.48 | 14 |
| B1 | 1.0 | 0.60 | 0.40 | 40 |
| B2 | 13.44 | 5.64 | 7.80 | 58 |
| B3 | 18.6 | 8.38 | 10.22 | 55 |
| B4 | 8.3 | 6.5 | 1.8 | 22 |
| B5 | 4.8 | 4.3 | 0.5 | 10 |
| E2 | 6.28 | 3.35 | 2.93 | 46 |
| E3 | 8.33 | 4.00 | 4.33 | 52 |
| E4 | 6.00 | 5.04 | 0.96 | 17 |
| E5 | 5.72 | 4.78 | 0.94 | 16 |

TABLE 2. - Gives enzyme activities in the fractions separated from the Bovine olfactory epithelium homogenate by discontinuous sucrose density gradient centrifugation. Specific activity is expressed as μ moles Pi released/mg protein per $\frac{1}{2}$ hr. (Reproduced from the M.Sc. thesis, A.Menevse, 1973.)

ATPase activities. Further fractionation of B₂ and B₃ resulted in several more fractions. E₂ and E₃ fractions from the last procedure also showed high enzyme activities. The relatively high activities of Mg²⁺-ATPase seems to have been derived from the high content of mitochondria in these fractions.

An attempt to separate different types of plasma membrane from the cow olfactory epithelia was, therefore, not successful. Findings of this kind are similar to those of Koch, 1969, and Koyama *et al.*, 1971. That is, the fractions obtained from the olfactory mucosa by subcellular fractionation techniques did not represent a pure olfactory receptor cell plasma membrane. Biochemical studies on the olfactory mechanism require a pure preparation of a receptor cell membrane fraction.

To obtain a pure membrane fraction - this could be achieved by isolating cilia from the dendrite part, i.e., the terminal swelling of the receptor cells. If the olfactory receptors are on the ciliary membrane which provides the means to put large areas of cell membrane into contact with odorous substances, then the cilia isolation procedure would be valid. Since, at the moment, there are no known distinctive biochemical markers for the plasma membranes of the cilia, the cilia in fractions from the mucosa could be characterised on the basis of their characteristic morphology using electron microscopy.

In protozoa, techniques have been developed to separate cilia, and even to separate their various components (Watson and Hopkins, 1962;

Gibbons, 1965). Moreover, only a simple technique was required to isolate pure rod outer segments from the retina of the frog (Bitensky et al., 1971). The rod is like a modified cilium and functions in visual reception.

The techniques described in the methods of this chapter have been employed for the isolation of cilia from the sheep olfactory mucosa.

The method used, which was as described for the isolation of the cilia from *Tetrahymena* (Method II), produced contaminating cellular debris as well as isolated cilia. Portions B, B₁, B₂ and B₃ representing each step of the method were separately examined in the electron microscope. It was found that portion B₁ contained more cilia than any other portion. Typical electron micrographs of this portion are shown in Figures 21 and 22. As can be seen, numerous cilia were collected in that fraction indicating the main step in the deciliation of the cilia from the cells. However, cellular debris was also present and subsequent centrifugation of this portion did not yield a pure cilia preparation. Other portions did not show a significant number of cilia (1 - 5% of the total). These cilia were sometimes without ciliary membranes.

The portion B₁ was the result of gently shaking the olfactory endoturbinates in a buffer and centrifugation of the suspension. Although it showed a high number of cilia in comparison to other

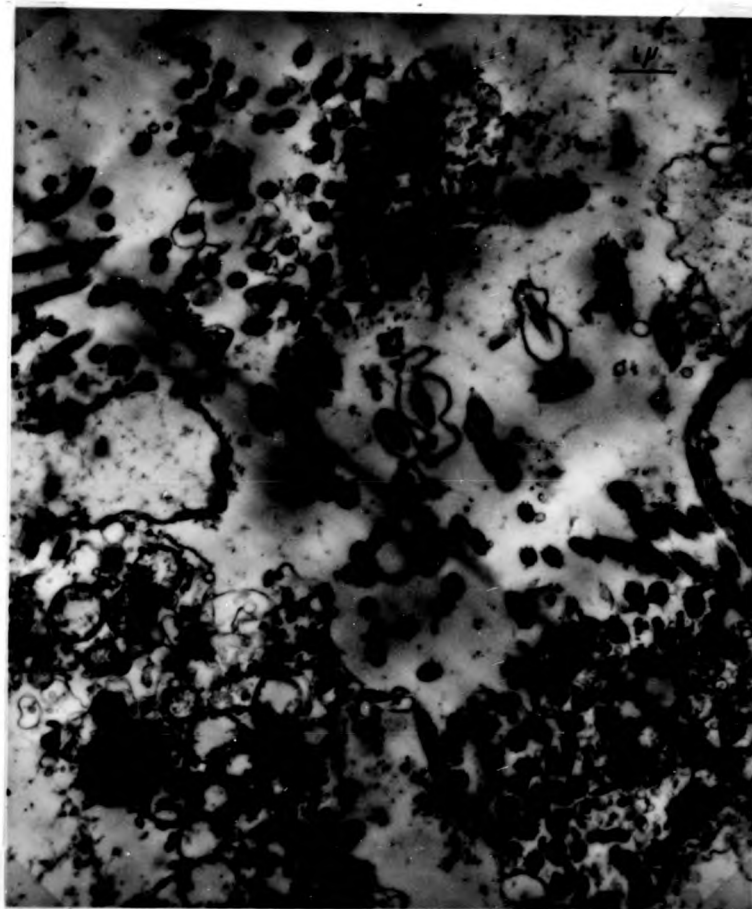


Fig. 21. The B₁portion obtained from the sheep olfactory mucosa according to method II. (see text) containing numerous cilia and cellular debris.



Fig. 22. Isolated cilia from the sheep olfactory mucosa found in the B, portion of Method II. (see text).

Negative staining with 1% Na tungstate.



Fig. 22. Isolated cilia from the sheep olfactory mucosa found in the B₁ portion of Method II. (see text).

Negative staining with 1% Na tungstate.

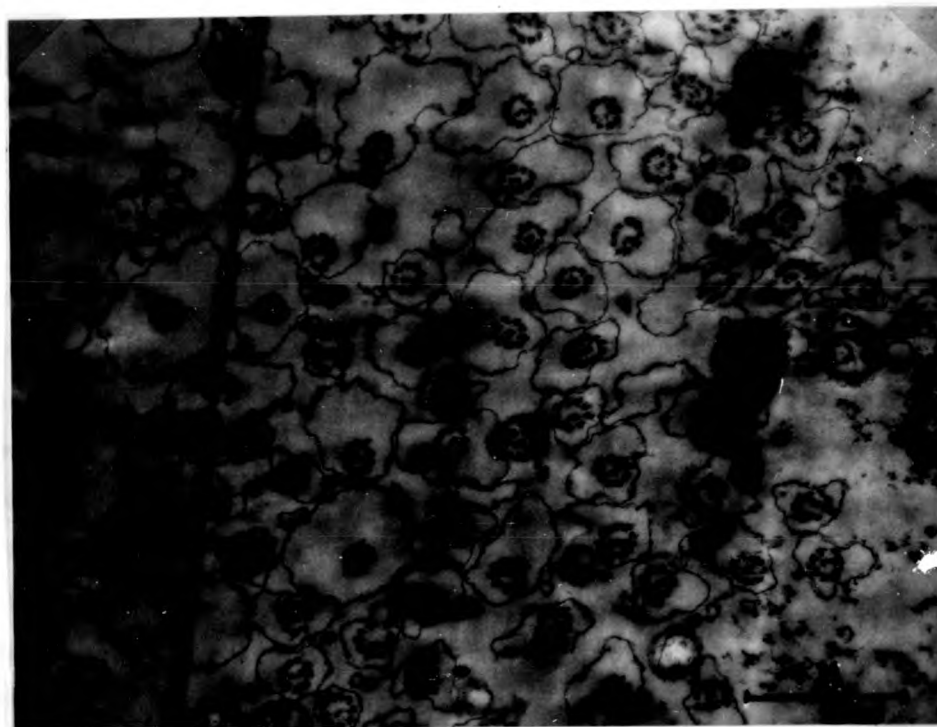


Fig. 23. Cross section of numerous cilia from the sheep olfactory mucosa, collected in the fraction B, of Method II. Negative staining with 1% Na tungstate.

portions, the percentage value of cilia occupied in this section of the portion did not exceed 30% of the total area examined. This was the result of 5 - 6 runs of experiments at different times with different olfactory tissue preparations. The cilia isolated in that portion were identical in terms of their microtubules and membranes. Cross-sections of cilia with expanded ciliary membranes can be seen in electron micrograph (Figure 23).

Method I, which was based on the vigorous shaking of the olfactory endoturbinates in 47% sucrose solution produced a striking result. The first portion, G₁, of this method contained isolated cells with numerous cilia. A typical electron micrograph showing the isolated cells is seen in Figure 24. The number of cilia per cell was around 100. The length of the cilium was 6.3 μ . With their morphological appearance, these cells can be likened to those of ciliated-columnar cells found in respiratory epithelia. Successive experiments using the olfactory epithelium from different sheep resulted in similar views of the G₁ portion in the electron microscope. These cells with their length of 40 μ are similar to those of the olfactory receptor cells (Graziadei, 1971)(Figure 25). However, their diameter is larger than that of olfactory neurons. They also lack terminal swellings (Figure 26). Examination of the thin sections from this portion confirmed the isolated cells as being from the olfactory mucosa. However, other cell organelles and debris were also observed. A finding of this kind is quite significant. About 50 years ago, Kolmer (1927) reported the replacement of part of the olfactory epithelium by non-

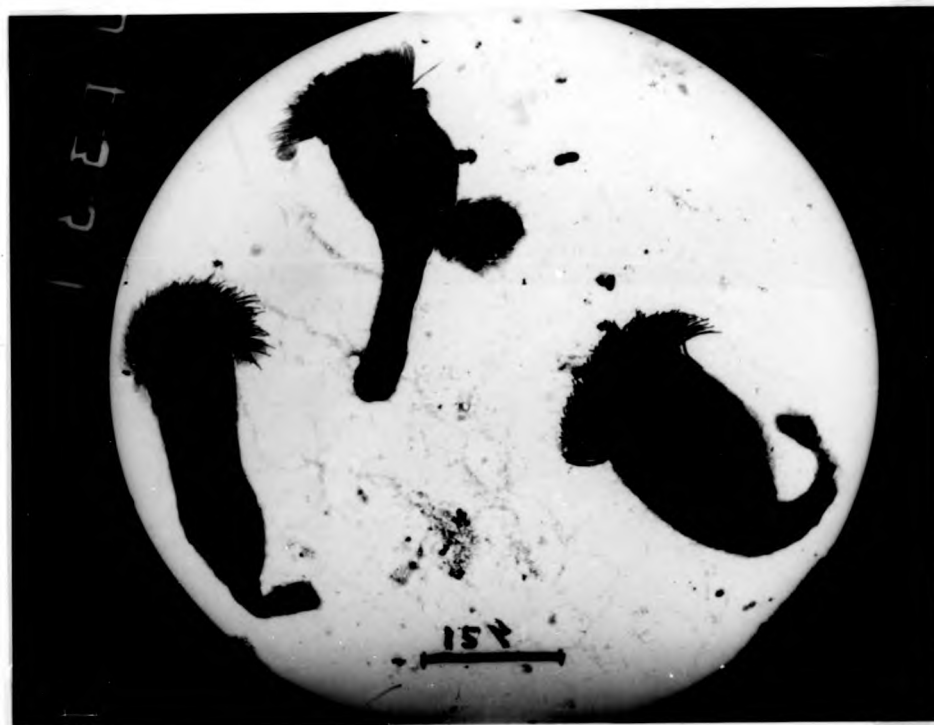


Fig. 24. Isolated cells obtained from the sheep olfactory mucosa
according to Method I. Negative staining with 1% Na tungstate.



Fig. 25. An isolated cell obtained from the sheep olfactory mucosa
according to Method I. Negative staining with 1% Na tungstate.

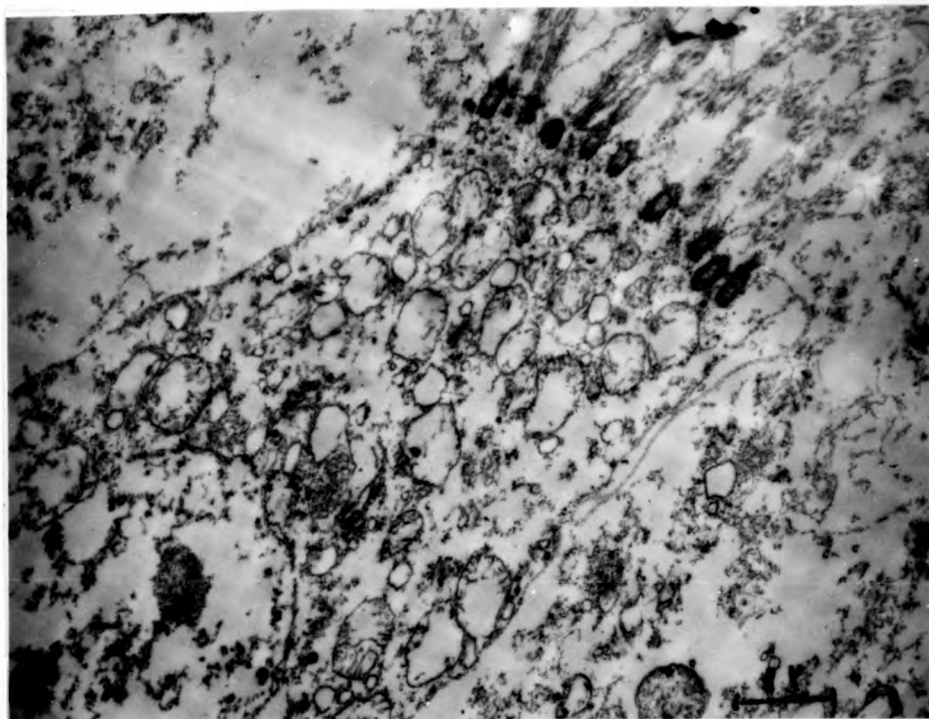


Fig. 26. Longitudinal section of the distal portion of the isolated cell obtained from the sheep olfactory mucosa according to Method I.

sensory ciliated respiratory epithelium in man as the result of disease.

Matulions (1975) has recently studied the effect of zinc sulphate on the olfactory mucosa of mice. He reported that olfactory sensory cells were regenerated after their destruction by the chemical. Although the underlying mechanisms for the regenerative process is not clear, the olfactory epithelium in the mouse apparently proceeds through stages in which "abnormally located cilia cells" are found. These cells with their shape and numerous cilia are similar to those found in the G₁ portion of the sheep olfactory epithelia in this work. They are reported to be non-sensory ciliated cells found in the olfactory epithelia (Matulions, 1975).

A finding of such ciliated non-sensory cells in the olfactory epithelia means that the isolated cilia do not entirely belong to sensory cells; they may well belong to respiratory cells. Therefore, any attempt to isolate the cilia from the olfactory mucosa would not be valid unless there is a morphological distinction between the cilia of olfactory neurons and the cilia of columnar respiratory cells. At the moment, there is no morphological difference between two cilia in different tissues and therefore morphological characterisation of olfactory cilia would be of limited utility.

CHAPTER 3

CHEMICAL MODIFICATION APPROACH TO OLFACTORY CODING

3.1 INTRODUCTION

Olfactory stimulation is induced by contact of odorant molecules in the external environment with the epithelium of the sensory neurons. From this aspect the reactions of the receptor cells serve as quantitative indicators of events at the receptor membrane. The brain requires information about the quality and quantity of the stimulus. These parameters are coded in the receptor signal i.e. the impulse pattern. Interaction of the odours with the presumptive receptors is similar to those of very important biological processes which involve the interaction of the stimulus molecule - the ligand, with the macromolecule - the receptor. The study of this interaction provides information about many aspects of the functions and mechanisms of biological processes. For example, enzymes catalyse reactions of their substrates and cofactors and the rates of these reactions may be changed by effectors; antibodies bind their haptens; hormones interact with their receptors; macromolecular carriers transport ligands across bio-membranes; and neurotransmitters react with their receptors. The location and isolation of receptors from highly complex systems, and the elucidation of the mode of action of the receptors with their ligands at the molecular level are some of the great problems facing molecular biologists.

Proteins and phospholipids are the major components of membranes. The basic question in olfaction is that of the nature of the olfactory receptor sites: are they protein binding sites or phospholipid binding sites? If either of these components play a critical role in the excitation phenomena, one would expect significant changes in excitability when the component is modified by chemical means. Such chemical modifications can be accomplished by using a variety of reagents that are known to react with specific group(s) of proteins.

It is accepted that the specific functions carried out by proteins are mediated by specialised regions on the macromolecules that are called the active sites. The active sites are three-dimensional structures adapted to their specific ligands (Singer, 1967). A large portion of the information concerning the chemical basis of enzyme action has been obtained through the application of protein modification techniques (Baker, 1967). In these techniques a chemical tag is covalently attached to some amino acid residue(s) within the active site so that, upon enzymatic or chemical degradation of the labelled protein molecule, peptide fragments originating from the active sites may be detected, isolated and characterised.

There are several approaches that may result in modification of proteins in the membranes. These can be classified as: 1) enzymatic modification of receptors, 2) modification with group-specific reagents, 3) modification with active-site-directed reagents (affinity labelling), and 4) photoaffinity labelling.

1) Enzymatic Modification of Receptors

Proteolytic and lipolytic enzymes have proved to be useful tools in elucidating structural features of membranes. For instance these enzymes have been applied in the study of photoreceptor membranes (Trayhurn et al., 1974). Treatment of bovine rod outer segment (ROS) membranes with pronase or subtilisin as proteolytic enzymes caused only limited digestion of rhodopsin. Rhodopsin remained spectrally intact and its regenerability was largely retained. However, SDS⁻ and C₁₂TAB⁺ gel electrophoresis and N-terminal determinations indicated that rhodopsin had a subunit structure, apparently caused by selective proteolytic action. Non-rhodopsin proteins, for instance, retinol oxidoreductases, are completely digested. Treatment of the membranes by either phospholipase C or detergents, followed by pronase incubation resulted in complete digestion, including loss of the 500 nm absorption of rhodopsin (Daemen et al., 1974). These results indicated that rhodopsin is an intrinsic membrane protein, which is only partly exposed to the aqueous phase external to the discs, whereas non-rhodopsin photoreceptor membrane proteins seem to be situated more externally.

Various hydrolytic enzymes have been used to selectively dissect nerve membrane components. The primary purpose was to identify the macromolecules responsible for conductance changes. Trypsin and α -chymotrypsin, when applied externally, were found to be ineffective in causing depolarisation and conduction block in lobster giant axons

and frog myelinated nerve fibres (Narahashi and Tobias, 1964). When applied to the inside of squid giant axons, trypsin and α -chymotrypsin blocked the conduction of ions. (Tasaki and Takenaka, 1964). Phospholipase C was found to be highly effective in blocking the action potential in squid axons (Tasaki and Takenaka, 1964).

Recently it has been shown that α -chymotrypsin has a special activity for the prolonged falling phase of the action potential in the squid giant axon membranes (Sevcik and Narahashi, 1973). Remarkable prolongation of the sodium inactivation was also brought about by internal perfusion of pronase (Rojas and Armstrong, 1971). These observations indicated that the part of the membrane protein susceptible to α -chymotrypsin or pronase is somehow related to the sodium inactivation mechanism.

2) Modification with Group Specific Reagents

In many enzymes, particular amino acid residues possess enhanced chemical reactivities. This often, but not always, related to the functioning of the residue in the catalytic act and derives from the particular local environment of the amino acid side-chain. That special environment causes a certain residue to have a much enhanced reactivity towards a reagent. For instance, the special environment of the active-site seryl residue in the serine proteases caused this residue to have a much enhanced reactivity, especially towards acylating agents (Koshland et al., 1966)

Many reagents are now available for specific modification of amino acid residues. Their properties, uses and potential have been discussed elsewhere (Glazer, 1970). Despite this recent proliferation of such reagents, few have received extensive study with regulatory systems. In view of the potentialities of such studies, it has recently been concluded (Pogson, 1974) that the present situation reflects again the problems of purification and obtaining sufficient quantities of protein.

Amino acid-specific reagents fall loosely into two groups, a) those which effect a change in molecular size, shape, catalytic activity or amino acid reactivity, all of which processes are monitored by standard techniques, and b) those which are themselves probes, possessing some reporter group which may be easily observed,

and which is responsive to conformational and other changes in the vicinity of the probe. Most reagents probably fall into the first of these groupings and are reactive towards thiol groups (Pogson, 1974).

Most of the thiol reagents are described generally as being specific but there are many instances where other functional groups can be attacked; this has been seen to be the case particularly with alkylating agents, e.g. iodoacetate, iodoacetamide, N-ethylmaleimide (NEM) (Knowles, 1974). In contrast, DTNB (5,5'-dithiobis-(2-nitrobenzoate) and similar disulphides appear to be specific for thiol groups; these reagents are particularly useful in that the free-SH group may frequently be regenerated by addition of small molecular weight thiols such as dithiothreitol (DTT). This reversibility allows protection of -SH groups while other functional groups are modified by less selective reagents.

The effect of treatment with the disulfide bond reducing agent DTT on the responsiveness of isolated strips of chick muscle to cholinergic agonists and antagonists has been studied (Rang and Ritter, 1971). After reduction, the muscle became less sensitive to drugs, carbachol and acetylcholine. The effects of reduction could be fully reversed by reoxidation with DTNB. These results, found to be comparable to those obtained on electroplax cells from Electrophorus electricus (Karlin, 1969), suggested that the receptor possesses a disulfide bond, reduction of which modifies the selectivity of the receptor without markedly affecting its function.

It has also been shown that the following reagents, 1-fluoro-2,4-dinitrobenzene (FDNB); 1,5-difluoro-2,4-dinitrobenzene (FFDNB), and 2,4,6-trinitrophenol (TNP) can suppress action potentials in lobster and squid axons. Furthermore, heavy metal ions, including those of mercury and silver and the more selective SH-reactive agents, NEM and P-chloromercuribenzoate (PCMB), can all reduce the electrical excitability of axons in proportion to their ability to form insoluble salts with sulfides. Their effects can be prevented or reversed with cysteine or glutathione (Huneeus-Cox, et al., 1966).

The loss of activity of a protein upon chemical modification can be due to modification of a residue within the active site (the modification can also be near the active site and still prevent the binding of the substance), or to secondary effects on the conformation of the active site having nothing to do with labelling the site. One way of discriminating between these two effects is the use of an unreactive, specific, competitive inhibitor, or protector, of the active site in question. If in the presence of the specific site protector, chemical modification of the protein results in a significantly smaller degree of inactivation than occurs in the absence of the protector, all other conditions being the same, this suggests that at least part of the chemical modification occurs within the active site.

Protection by substrates or related compounds has been used to selectively prevent the modification of active-centre residues in many enzymes (Singer, 1967).

3) Affinity Labelling

Affinity labelling is a technique by which protein binding sites may be covalently labelled with a small ligand substrate (Singer, 1967; Shaw, 1970). This involves the prior incorporation of a reactive group into the ligand structure such that, when the ligand is presented to its specific binding protein, it may still interact reversibly, but subsequent reaction within the complex will serve to attach it irreversibly and covalently to amino acid residues at, or near, the active site. The affinity labelling technique mainly involves as the irreversible step an alkylation and acylation reaction (Singer, 1967; Shaw, 1970; Kiefer et al., 1967).

Recently, Knowles (1974) has argued the matter in detail and said that the ideal active-site-directed inhibitor should satisfy three points.

- 1) It must contain the structural element required for binding at the active site.
- 2) It must not react rapidly with water, nor too vigorously with the protein.
- 3) For an enzyme, the reagent should, with benefit, be designed in the knowledge of the probable catalytic mechanism.

Enzymes, which can be obtained essentially pure, have been extensively studied by affinity labelling techniques. Kallos and Shaw (1971) labelled an allosteric site in glutamate dehydrogenase

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using bromoacetyl diethylstilbestrol. Chin and Warren (1968) used the 4-mercuri derivative of estradiol to label binding sites on glutamate dehydrogenase.

The specificity of an antibody for its antigen is similar to that of an enzyme for its substrate. It is not surprising, therefore, that the affinity-labelling technique was applied very early on, and with considerable success, to the determination of residues in the antibody combining sites (Metzger et al., 1968). When hapten-specific immunoglobulins were treated with diazonium salts closely resembling their particular hapten, a reaction occurred which thereafter prevented the binding of hapten. The presence of hapten or close analogs prevented the action.

4) Photoaffinity Labelling

In the past ten years much interest has centred around the technique of photoaffinity labelling. In this variation of affinity labelling the attaching group is light-sensitive and reacts only when irradiated with UV light.

Westheimer's early work on enzyme active sites with diazo esters introduced the technique of photoaffinity labelling (Singh *et al.*, 1962), but few reports appeared until the late 1960's. Knowles (1972) stated that it might be dangerous to use reagents selective for nucleophiles, since the affinity labelling technique relies on local concentration differences and misleading labelling patterns may arise in which the nearest accessible nucleophile is tagged. Furthermore, any approach that is based on a reagent used in aqueous solution is limited by the fact that water is more reactive than many of the functional groups in amino acid side chains. Bearing in mind the limitations of conventional affinity labelling, Knowles looked for a reagent capable of attacking even carbon-hydrogen bonds at the specificity site. Clearly such a species could not be added externally to an aqueous solution of protein, and must be generated from a stable precursor in situ. The only chemical intermediates capable of C-H bond insertion are carbenes, nitrenes and other similar reactive species. These can be generated photolytically. The precursor must be: a) chemically inert in the dark, b) photolysable to a species that does not rearrange intramolecularly to a less

reactive entity, and c) susceptible to photolysis at wavelengths well clear of protein absorption.

Three photosensitive functions have been used in the study of binding sites: 1) aryl azides, 2) diazoketones, diazoesters and 3) diazirines. Arylazides and diazoketones have been used by Fleet et al (1972), and Richards (Converse and Richards, 1969) to study binding sites of antibodies.

There are no really stable photo-precursors of carbenes, but, as nitrene precursors, aryl azides have the required chemical stability. It is, therefore, in the past two years that the number of papers concerning the use of aryl azides as nitrene precursors, have shown a dramatic increase.

However, it has been reported that photo affinity labelling by nitrenes may be more complex than hitherto supposed (Ruoho et al., 1973). In an attempt to minimise non-specific labelling of erythrocyte acetylcholinesterase (AChE) by the nitrenes derived from two aryl azides, 4-azido-2-nitro-benzyltrimethylammonium and 4-azido-2-nitrotriethylammonium (HK-83), irradiations were carried out in the presence of 4-aminobenzoate, a scavenger for those photolysis products of the azides not formed at the receptor site(s). It was found that 4-aminobenzoate and soluble proteins prevent the photo-inactivation of AChE by the azide. Furthermore, HK-83 effectively inhibited AChE when irradiated before addition to the enzyme, but if

the pre-irradiation was carried out in the presence of 4-amino-benzoate the labelling became non-specific. The authors call this effect "pseudo-photoaffinity labelling". True photoaffinity labelling occurs only when the rate constant(s) for reaction of the bound photogenerated species at the receptor site(s) are significantly larger than the rate constant for dissociation of the species from the site(s).

However, aryl azides as photoaffinity labelling reagents have been successfully applied to complex ligand-receptor interactions. An interesting piece of work concerning this interaction has provided evidence that 8-N₃cAMP would prove useful as a photoaffinity membrane probe for cAMP binding sites (Haley, 1975).

Studies on electron transport systems with the photoaffinity labelling uncoupler 2-azido-4-nitrophenol (NPA), have shown that the mitochondrial uncoupler-binding sites are located exclusively in Complex V. Complexes I, III and IV, which carry the three coupling sites of the respiratory chain, had negligible capacity for the binding of NPA, whereas the uncoupler-binding capacity of Complex V appeared to be increased two- to threefold as compared to whole mitochondria (Hatefi et al., 1975).

The above findings have led to the conclusion that photoaffinity labelling, as opposed to conventional affinity labelling, would be more desirable as a tool for studying odour-receptor binding.

In this work, several aryl azides were synthesised to be used as photoaffinity labelling reagents in the detection of odour receptors by an experiment associating electrophysiological and biochemical techniques. Using the same technique, group-specific protein reagents were applied, in both vapour and liquid phases to the olfactory eminence of the frog to determine the nature of the receptor sites for odorants. Protection experiments, analogous to substrate protection of an enzyme from inhibition, were carried out with an odorant in solution as the protecting agent and a group-specific reagent as the inhibitor. One of the studies made in this part of the work was to find out if there are different receptors for different classes of odorants. Attempts have also been made to determine whether: a) olfactory receptors, if they are proteinaceous, could be eliminated by proteolytic enzymes; b) the location of olfactory receptors differs topologically for different classes of odorants; c) the effect of tritonX-100 and phospholipase C as membrane lipid reagents would change the responses evoked from the olfactory mucosa upon odour stimulation. It was the general purpose of this work to see if modification by chemical means could be an approach to the olfactory coding mechanism.

3.2 MATERIALS AND METHODS

A. EOG Recording

1. Animal Preparation

These experiments were performed on the common frog, Rana temporaria, obtained from Gerrard Biological Supply (East Preston, West Sussex). They were kept for a period of days in a cold room (+4°C) after delivery, and each frog was warmed at room temperature for at least 2 hours before each experiment.

The frogs were first anaesthetised by immersion in 15ml urethane solution (10% w/v in H₂O), absorption being arrested by rinsing the skin with tap water as soon as the reflex responses to eye poking and skin tweaking were abolished. The frogs remained anaesthetised by this treatment for the whole course of the experiment and they were not allowed to recover.

The olfactory eminence on one side was exposed by cutting away the skin, cartilage and finally the epithelium of the roof of the nasal cavity, great care being taken to prevent any spillage of blood on to the eminence. The frog was covered with a moist cloth and fixed by ear bars in a perspex frame, which was screwed in position beneath the adjustable odour applicator and electrode holders. A good blood circulation was found to be essential for stable EOG recordings.

For the experiments in which solutions of reagents and n-amyl acetate were applied onto the olfactory eminence, a Teflon strip was used to block the buccal aperture of the internal naris. This prevented the drainage of solutions from the olfactory cavity.

2. Electrodes

The reference or ground electrode was a AgCl-Ag wire coiled at one end, wrapped in muslin, soaked in 0.9% NaCl (Saline) and inserted in the animal's mouth. The exposed length of silver wire was sealed in Teflon tubing.

The EOG or recording electrode was a micropipette, pulled from Pyrex (lmmi.d. tubing, to produce a smooth tip of diameter 30 - 100 μ . It was filled with either normal saline or 3MKCl containing 2% agar. For the experiments in which protein reagents were used, agar was not utilised, the electrode was only filled with saline or 3MKCl to prevent any possible side reaction between the agar and the protein reagents. Electrical connection was through a AgCl-Ag wire inserted in the pipette. Each electrode had a resistance of about 0.3 - 1.0 megohms, and lasted for 3 - 4 experiments.

All pipettes were examined under a low power microscope before each experiment to ensure that the filling process had been carried out correctly.

3. Electronic Equipment

The Keithley Model 610B Electrometer was used to amplify the signals picked up by the electrodes. It is a DC amplifier with an input resistance greater than 10^{14} ohms.

The amplified signal was plotted by a potentiometric chart recorder (Smith's Servoscribe).

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1. Chemical

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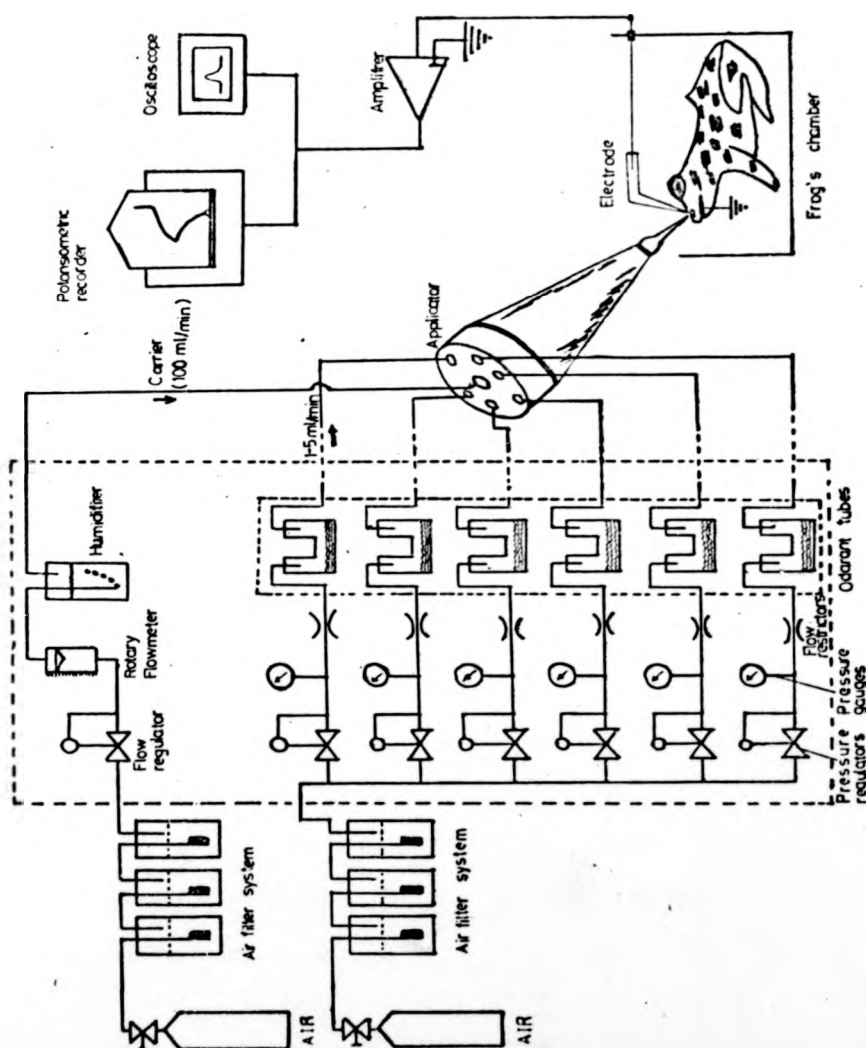


Figure 27. Stimulating and Recording Apparatus for E.O.G.'s.

B. Olfactometer

The olfactometer with its stimulation system and applicator described in Figure 27 is similar to that of T. M. Poynder (1974).

1. Chemicals and Solutions

The chemicals used in these experiments included:

N-ethylmaleimide (NEM); 1-fluoro-2,4-dinitrobenzene (FDNB); 2,4,6 trinitrobenzene-sulfonic acid (TNBS); 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB); (these were purchased from Eastman Kodak Co., Ltd.); mersalyl{O-[3-(hydroxymercuric)-2-methoxypropyl] carbonyl} phenoxy acetic acid sodium salt; p-chloromercuribenzoic acid (pCMB); dithiothreitol (DTT); tritonx-100; phospholipase C (from the Sigma Chemical Co., Ltd.), and Nbf-Cl (4-chloro-7-nitrobenzofurazan) (this was purchased from Aldrich Chemical Co., Ltd.). All chemicals were of the highest purity obtainable, and used without further purification.

The odour stimuli used in the vapour phase were: n-amyl acetate; 1,8 cineole; m-xylene; phenylacetaldehyde (these were purchased from the British Drug Houses Ltd.), ethyl n-butyrate; naphthalene; butyric acid (obtained from Aldrich Chemical Co., Ltd.), n-butyl acetate; butanol; nitrobenzene; β -ionone; benzaldehyde (purchased from Fison's Chemical Co., Ltd.), linalyl acetate was a kind gift from Hoffman - La Roche, Inc. All chemicals were analytical reagent grade. The stimuli were refreshed for each experiment.

Frog's chamber



Figure 27. Stimulating and Recording Apparatus for E.O.G.'s.

The odorants used were selected on the basis of various criteria:

- a) an effective stimulus for the frog,
- b) detectable by the monitoring device at the lowest concentration applied,
- c) available in high purity,
- d) innocuous to the olfactory epithelia,
- e) stable at room temperature.

Enzymes used in this part of the work were: trypsin (2 x crys.), protease (repurified type VI); they were obtained from the Sigma Chemical Co., Ltd. Pronase B grade was purchased from Calbiochem. Chemical Co., Ltd. Crystalline bovine serum albumin was obtained from the Sigma Chemical Co., Ltd.

All solutions were made up in double distilled water. Amphibian Ringer's^{solution} was used for experiments concerning protein reagents and drugs. This Ringer's solution had the following composition: 111mM NaCl; 1.9 mM KCl; 1.1 mM CaCl₂; 24 mM NaHCO₃. The pH of all solutions was 7.0. However, when acidic reagents were dissolved in Ringer's the pH of the solution was adjusted to 7.0 with 0.1 M NaOH. In some enzymatic modification studies (mentioned along with their rationale), the pH of solution varied. FDNB is insoluble in Ringer's. Therefore this chemical was dissolved in reagent-grade methanol, and the solution was added dropwise to Ringer's with vigorous stirring to a final methanol concentration of 0.5% (v/v). A solution of 0.5% methanol in Ringer's was used as the control buffer for the experiments

involving this reagent. The same solution was also used for the protection experiments where n-amyl acetate was applied in liquid phase. pCMB was dissolved in 0.1M glycylglycine buffer. This was added to an equal volume of Ringer's, the pH of the resulting solution was 8.0. The control buffer for this series of experiments was a 1:1 (V/v) solution of the buffer and Ringer's: the pH was adjusted to 8.0.

Solutions of enzymes and reagents were always freshly prepared on the day of use. Liquid paraffin was used to dilute the odorants.

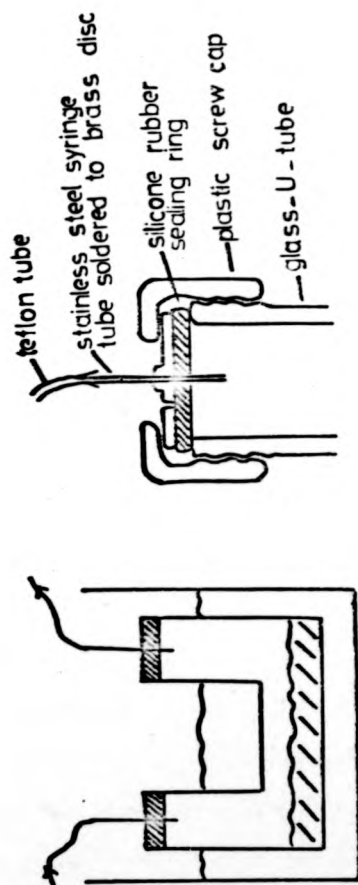


Fig. 28 A type of odorant tube with details of sealing.
Further explanation in text.

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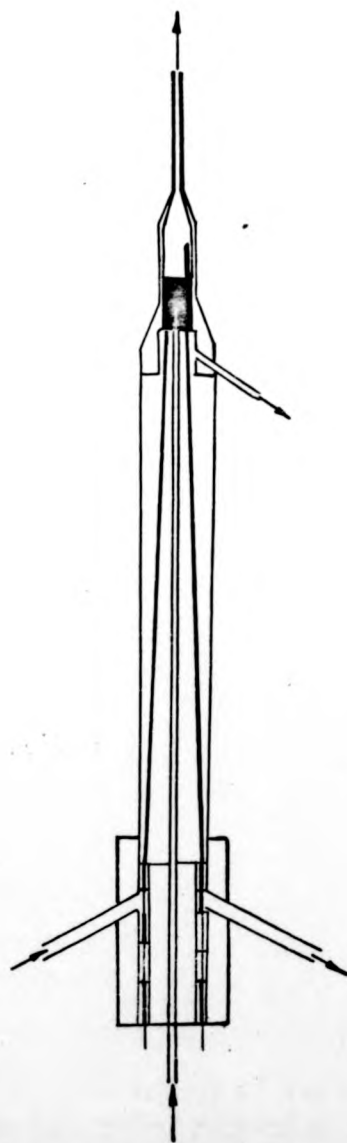
2. Odorant Flow Control

1 - 2 ml of the odorant, or odorant solution, was held in one of the specially constructed U-tubes, through which a steady stream of clean air (1 - 5 ml/min.) was passed. These U-tubes were immersed in a water bath, held below room temperature to prevent condensation. Considerable attention was paid to the connections with the odorant tubes. With the final arrangement shown in Figure 28, of the three parts which come into contact with the odorant, the silicone rubber sealing disc is cleaned with acetone and dried after use, while the glass and metal parts, after washing thoroughly in appropriate solvents, are dried at above 150°C. The Teflon tubing connecting the odorant tubes to the applicator has air passing through it except during experiments and seldom needs to be renewed.

The odorant streams (up to six in number) are generated by passing clean dry air over pools of liquid odorants held in U-tubes. These U-tubes have a straight central portion so that the air stream passes over about 8 cm² liquid surface without bubbling through it. This prevents formation of a spray which might be carried forward and thus upset the concentration. The air flow through each tube can be regulated from about 1 ml/min. to 5 ml/min. by controlling the pressure with a sintered stainless steel flow restrictor before the U-tube (5 - 25 p.s.i.). The odorant flow was diluted with a constant air flow of 50 ml/min. in the applicator (see next section), altering the pressure altered the dilution factor from about 1:50 to 1:11.

Figure 27 also shows the carrier air supply to the applicator. This carrier air was washed and humidified with deodorised water before being led by Teflon tubing to the applicator. A constant flow valve was used for the carrier air, and the flow rate was monitored directly with a rotary flow meter. This constant stream (50 ml/min.) of clean moist air plays continuously on the olfactory epithelium. This prevents the mucus from drying, prevents extraneous odours and, when required, carries the odour stimulus to the sensory area.

Figure 29. Scale drawing of six-channel odour applicator.
(Sagittal section channel 1 off, channel 4 on.)



3. Stimulus

The main channel odour is described by

The function of the device is to be added and of predetermined nasal cavity consists of a mechanism which directs the flow of air. The device is shown in Figure 29. The principle of operation is one for each channel. The device which leads to the orifice of the channel is a movable, either to the orifice of the channel.

The six channels are arranged symmetrically of the central channel. The six radial channels are shown in the above-mentioned drawing.

3. Stimulus Switching

The main part of stimulus switching is the applicator. A six-channel odour applicator was used. This device has previously been described by its designer, T. M. Poynder (1974).

The function of the applicator is to enable odourant streams to be added and mixed with the main carrier stream so that the odour stimuli of predetermined duration and sequence can be directed into the frog's nasal cavity. The applicator, which is illustrated in Figure 29 consists of three parts. These are the stream switching part, the mechanism for operating the switches and the nozzle which mixes and directs the gas stream towards the animal. The switches and nozzle are shown on a larger scale in Figure 30. There are six switches, one for each odourant stream. Each switch operates on the following principle. The carrier gas divides at a T junction one branch of which leads to a vent while the other leads to the nozzle and the frog. An odourant is introduced into one or other branch of the T by means of a movable, loose-fitting inner tube. It is swept by the carrier air, either to the vent or to the nozzle according to which branch the orifice of the odourant tube has moved into.

The switches are designed so that each can be actuated independently of the others. Carrier gas flowing at 100 ml/min. splits into six radially disposed ducts. Each of these then divides at one of the above-mentioned T junctions. Therefore 100/12 ml/min. gas issues

Figure.31. Pneumatic pistons of 6-channel applicator showing channel 1 'off' and channel 4 'on'.

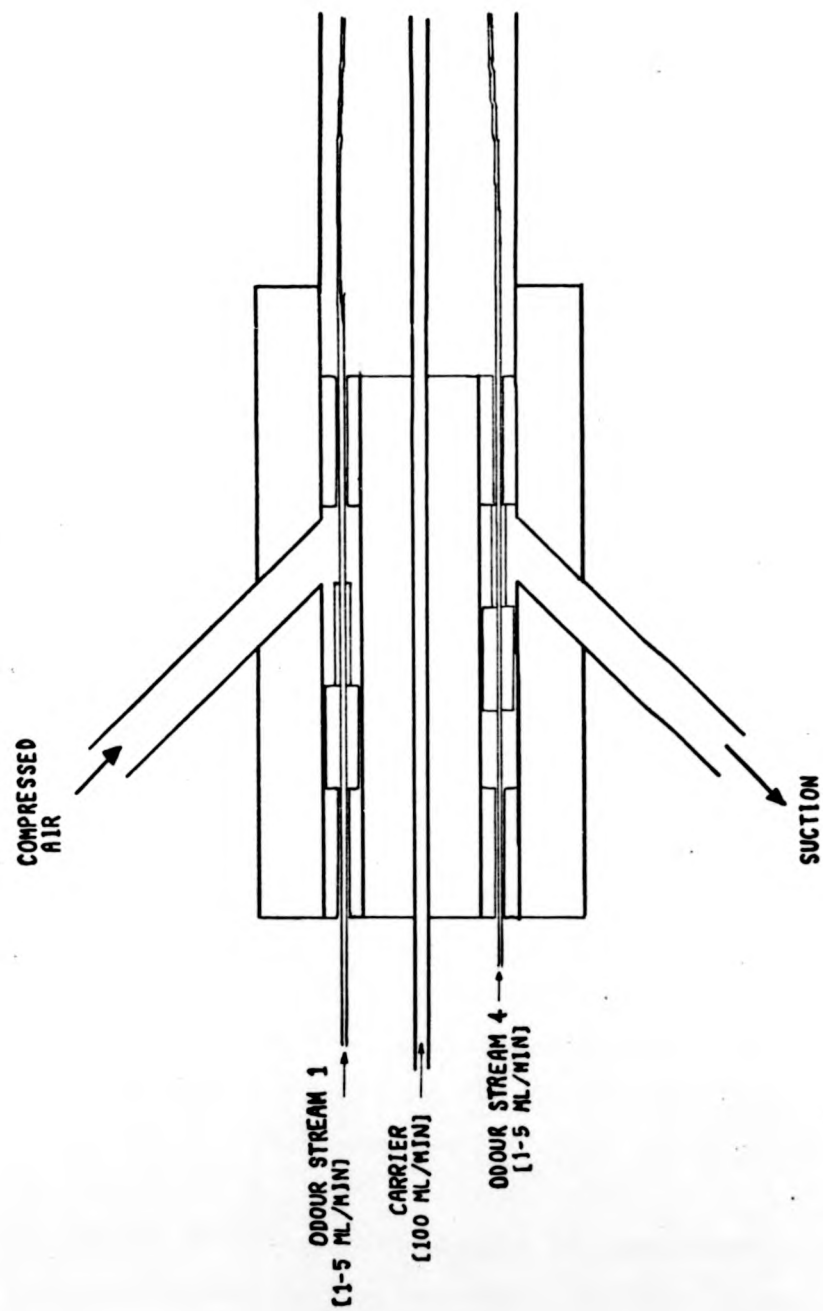
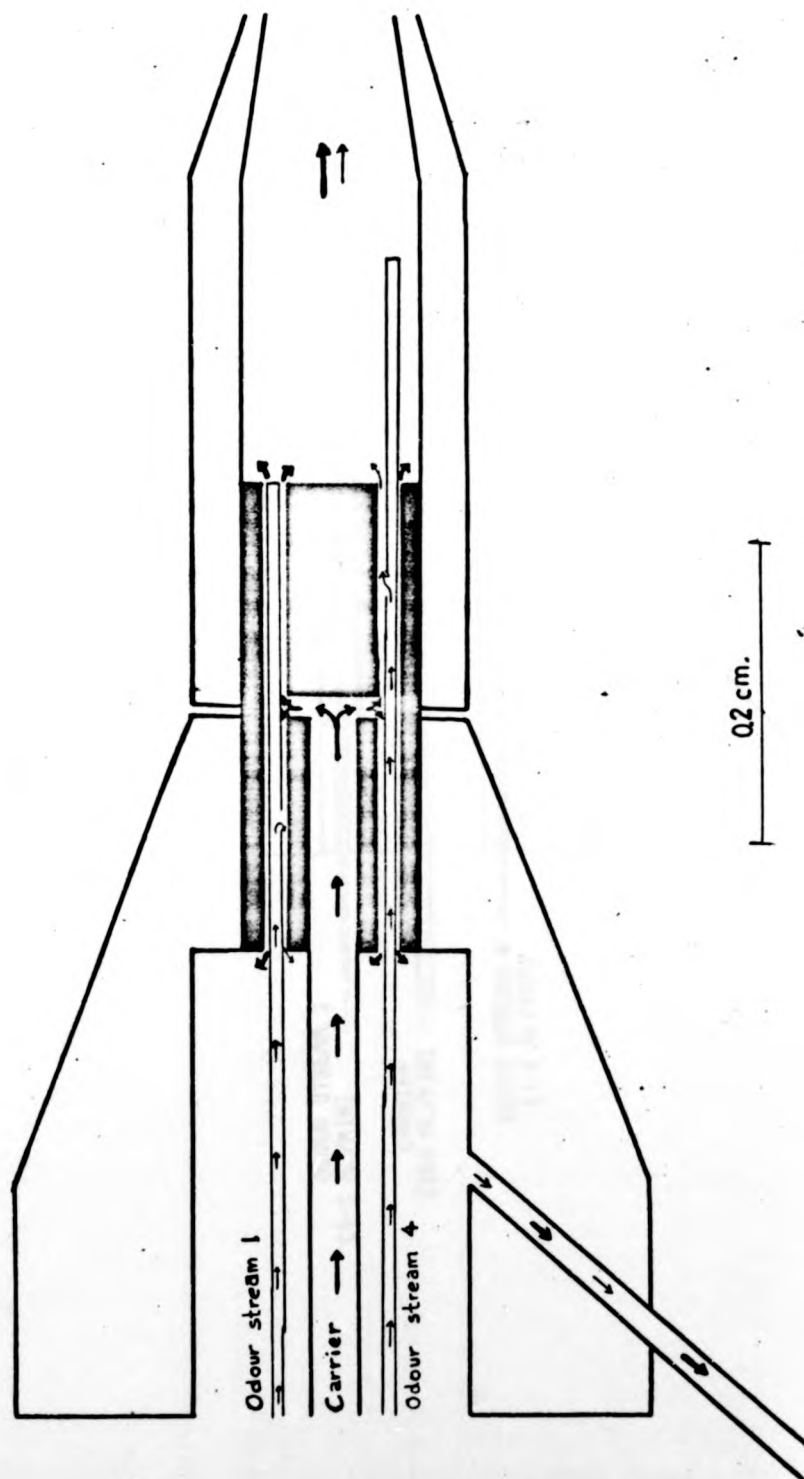


Figure.30. Detail of applicator stream switching system, showing channel 1 'off' and channel 4 'on'. (Glass nozzle on right is not all shown.)



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from each of the 12 branches of the T's. Six of these recombine as they enter the nozzle which therefore delivers 50 ml/min, while the other six lead away to a vent. Odorant streams not being applied and escaping at the vent are prevented from entering the room by an extraction duct.

The movable, loose-fitting inner tubes which convey the odorant into branches of the T's are stainless steel tubes of outside diameter 0.3mm. They are moved parallel to their axes by means of pistons which are fixed to them and which run in cylinders. The cylinders are each connected at one end to a source of compressed air or to suction. The change from compressed air to suction is made by solenoid valves which are activated automatically (Figure 31).

0.2 cm.

3. EXPERIMENTAL PROCEDURES

The frog was prepared as described under Animal Preparation. The applicator was positioned with its nozzle pointing into the opened olfactory cavity and about 5 mm away from it. The recording electrode was lowered by means of a micromanipulator so that its tip just touched the surface of the mucus overlying the olfactory eminence. Three test puffs of the stimulus were delivered and their EOGs were recorded to determine whether the receptors were responding normally. The stimulus intensity was kept constant as described in section B - 2. The odour dilution factor for the odorants was determined in order to work on a concentration which would give a half value (I/50) of the ~~maximum~~ EOG amplitude to a particular odorant. This was done by lowering the odour pressure from 25 p.s.i. to 5 p.s.i. value.

All stimulations were between 5 - 10 seconds in duration; an interval of 2 - 3 minutes was allowed for recovery between each stimulation.

For the liquid phase experiments in which proteolytic enzymes, group specific protein reagents or n-amyl acetate were applied onto the olfactory eminence, the following procedure was carried out: after delivery of the test puffs, the recording electrode was lifted by means of a micromanipulator, and its tip placed in a beaker of Ringer's. The olfactory cavity was filled with a physiological buffer solution, or Ringer's or phosphate buffer. The olfactory eminence was always

completely covered with solution. One c.c. disposable syringes with stainless steel needles were used to fill and drain the olfactory chamber. Separate syringes and needles for filling and draining were gently introduced into and withdrawn from the cavity with a needle to one side of the olfactory eminence to avoid mechanical damage to the tissue. The solution was allowed to remain in the cavity for a specified time period (usually 10 minutes); it was then changed (i.e. the solution was drawn off and replaced with fresh solution) firstly immediately after filling and then at the end of 5-minute intervals. After withdrawal of the physiological solution, the recording electrode was lowered to the same spot as for the test puffs, and three EOGs were elicited. These responses were used as the control responses. The micropipette was again lifted and its tip was placed in Ringer's.

The olfactory cavity was then filled with solution of the protein reagent or the enzyme solution in the Ringer's or the buffer used initially; this solution was changed in the same pattern as the control solution. After a specified period of time, this solution was withdrawn, and three EOGs were elicited to determine the effects of the reagent on this response.

To test the degree of reversibility of the effects of the reagent, the electrode was lifted and the cavity was again filled with the Ringer's for a given period of time with the same pattern of solution changes as described above. This step of the procedure will be

referred to as "washing" the mucosa. The cavity was then drained, the electrode was lowered and three EOGs were elicited.

In some experiments, the solution containing the reagent or enzyme was left on the mucosa for one to three minutes, then withdrawn, and one EOG was elicited. This procedure was repeated until the response was abolished or reached the level where EOGs had the same amplitude. In this way, the time course of development of the effects of the reagent could be followed.

More detailed experiments, along with their rationale, will be discussed under Results.

3.4 RESULTS

Solutions

Amphibian Ringer's was used as a solvent for the proteolytic enzymes and group specific reagents. In all experiments, the averaged amplitude of at least 3 EOGs (they were similar in terms of mV amplitude and waveform) elicited after an initial exposure to Ringer's for the same period as that required for exposure to the reagent or proteolytic enzyme, was taken as 100%, subsequent experimental values were compared to the averaged value. The effect of Ringer's solution on the EOGs to all odorants used in vapour phase in this work was found to be small. The original EOG value decreased to 75% and recovered its original amplitude after 2 - 3 stimulations with the odorant.

Ringer's + 0.5% MeOH was used as a solvent for the odorant, n-amyl acetate. In the protection experiments n-amyl acetate was dissolved in this solution. The effect of this Ringer's + 0.5% MeOH on the EOGs to the odorants was similar to that of Ringer's. It had little effect, decreasing the EOG amplitude by about 10 - 25% in all experiments, followed by recovery as above.

Repeated exposure to both Ringer's lasting from 1 minute to 20 minutes again had little effect, decreasing the EOG peak amplitude by 10 - 20% of the original value. The EOG waveform was not affected.

Ringer's at pH 8.0 also had little effect on the EOG responses to the odorants. The effect was the same as that of Ringer's pH 7.0.

Phosphate buffer (0.1 M) of pH 7.0 was used as a solvent for the enzymes, trypsin and pronase. This buffer had the following relative ion concentration: 117 mM Na⁺, 37 mM K⁺. Repeated applications of this buffer caused an increase of 5 - 10% of the original EOG peak amplitude. The initial rate of rise was unaffected, but the rate of decay was slightly reduced when n-amyl acetate and 1,8 cineole were used as stimuli.

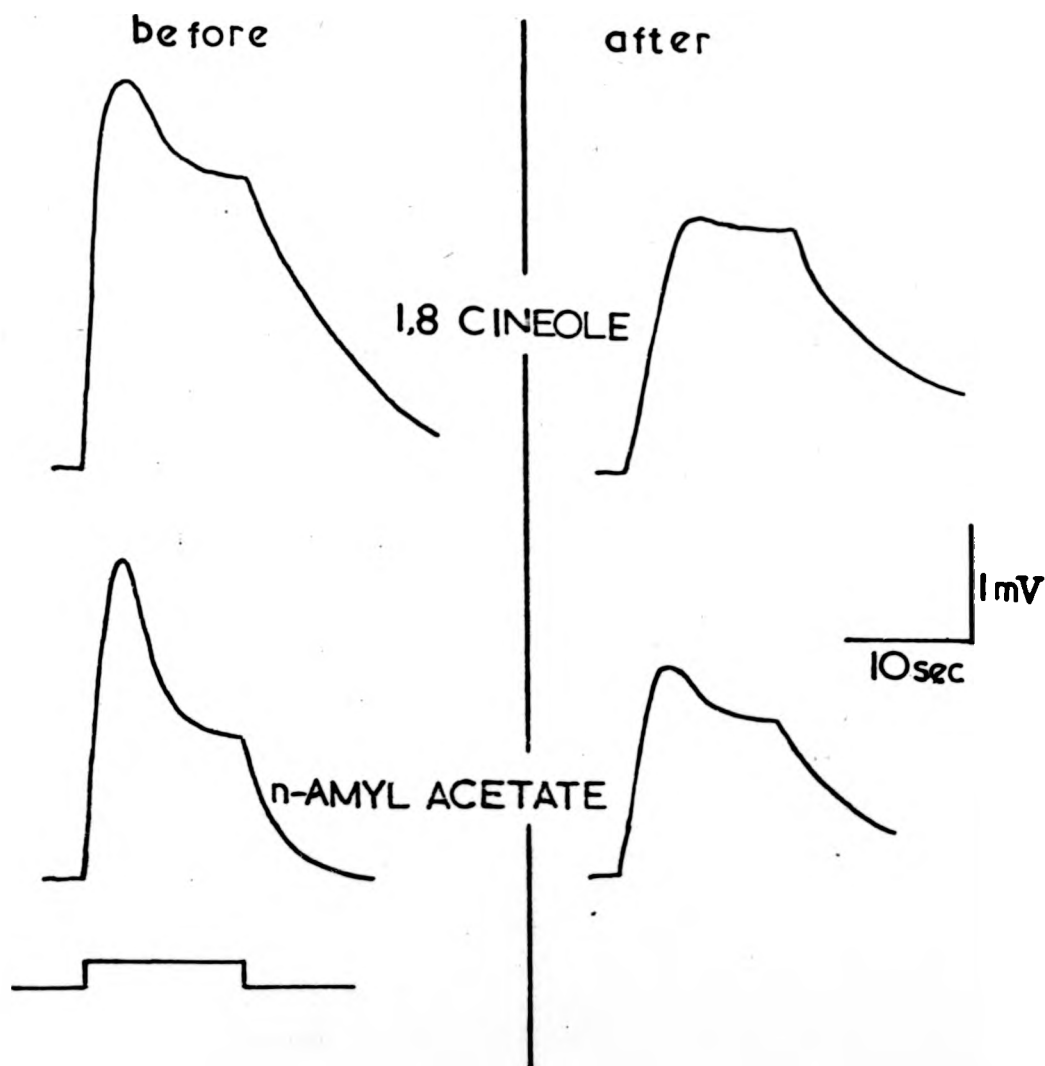


Fig. 32. Effect of Trypsin (5 mg/ml in Ringer's PH 7 for 5 min.) on the EOG's elicited by 1,8 cineole and n-amyl acetate.

3.5 RESULTS - P

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3.5 RESULTS - PART A

Enzymatic Modification Studies

Applications of proteolytic enzymes either in Ringer's or in phosphate buffer (pH 7.0) had similar effects on the EOGs elicited by n-amyl acetate and 1,8 cineole. That is, the effect of proteolytic enzymes is not solution dependent. All enzyme modification studies were carried out at room temperature and the incubation process was performed by introducing ^{the} enzyme solution into the frog's olfactory cavity which had already been converted into a water-tight "reaction chamber".

It has been noticed that after every enzyme digestion treatment of the frog's olfactory eminence, the EOG amplitude to n-amyl acetate was affected more than that of 1,8 cineole. This effect is generally on the ^{EOG} peak amplitude to the odorant. The rate of rise of the slow potential was not affected but the rate of decline was prolonged.

TRYPSIN

In concentrations ranging from 5 mg/ml to 10 mg/ml in either Ringer's or phosphate buffer (pH 7.0), this enzyme slightly reduced the EOG peak amplitudes to both n-amyl acetate and 1,8 cineole. This reduction was about 20 - 30% of the original EOG value, on the EOG peak amplitude. The EOG plateau was not affected. Therefore, the peak to plateau ratio was reduced by the treatment. A typical result of this experiment is shown in Figure 32, in which before the

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for 5 min.) on
amyl acetate.

treatment, the EOG peak to plateau ratios to n-amyl acetate and 1,8 cineole are 2.33 and 1.38 respectively, whereas after the treatment, the peak to plateau ratio to n-amyl acetate was reduced to 1.34. This value for 1,8 cineole was 1.06.

SEMI-ALKALINE PROTEASE

Incubation of the olfactory eminence with the semi-alkaline protease (pH 8.0, 10 mg/ml, in Ringer's) reduced the EOGs to n-amyl acetate and 1,8 cineole as two different classes of odorants, floral and camphoraceous respectively. The effect was found to be the same in three frogs, in each case, the EOG peak amplitude to odorants was first decreased to the level of the plateau,^{and} there was then no difference between the peak and the plateau. The decrease of the EOG amplitude continued with the increase in time. In all three experiments, it was noticed that after 15 minutes incubation and 10 minutes washing time, there were decreases in the level of the EOG amplitude. The amplitude of the EOG elicited by a puff of n-amyl acetate was reduced by about $81\% \pm 8\%$ (mean \pm S.E.M. of 3 experiments) at the end of recording. The reduction for 1,8 cineole was $75\% \pm 7\%$ (mean \pm S.E.M. of 3 experiments). The effect of semi-alkaline protease was partially reversed by the washing procedure (with Ringer's, pH 8.0 for 10 minutes) bringing up the responses to $50\% \pm 11\%$ for n-amyl acetate and $55\% \pm 12\%$ for 1,8 cineole. The effect of semi-alkaline protease on the EOG responses to n-amyl acetate and 1,8 cineole is shown in Figure 33 .

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Fig. 33. Effect of semi-alkaline protease on the EOGs to the odorants. Broken lines indicate the partial recovery of the responses after the washing procedure. (mean \pm S.E.M. of 3 exp.)

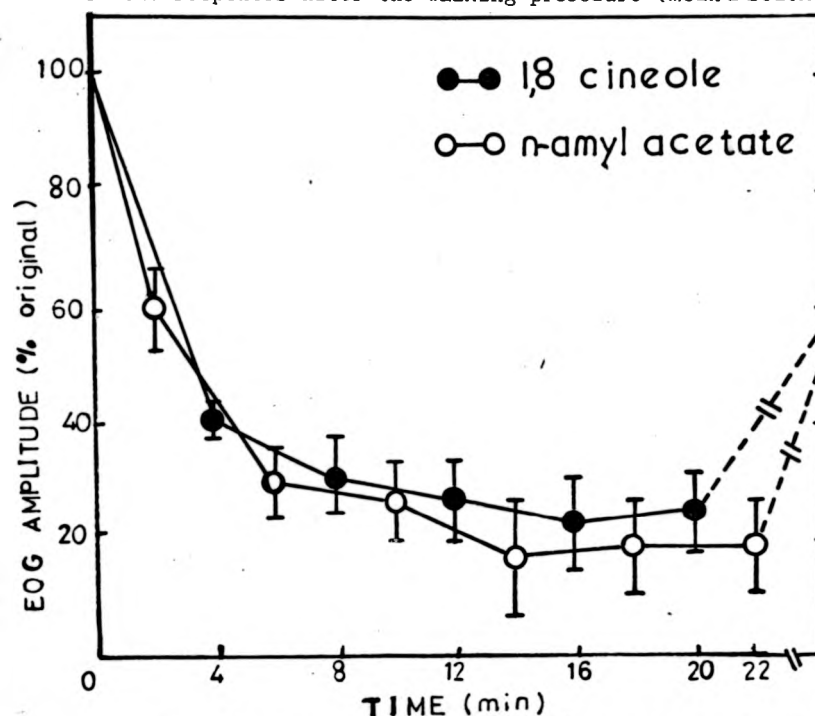
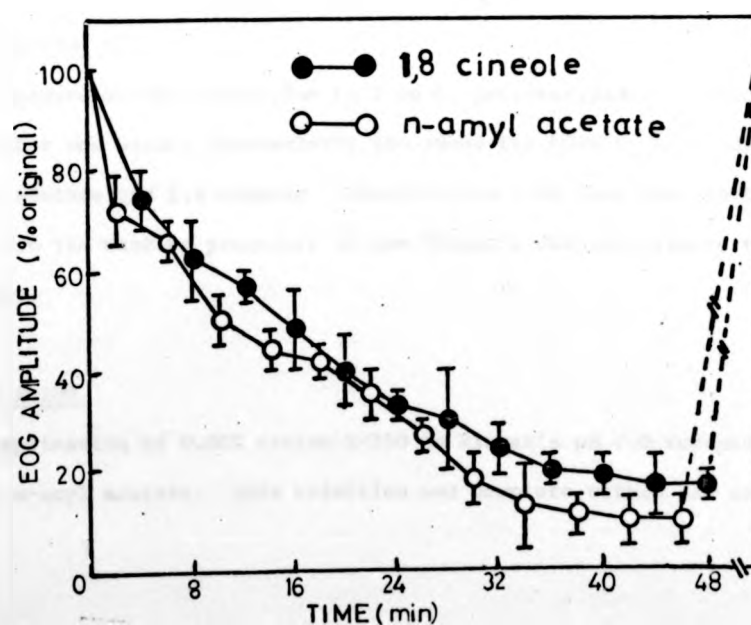


Fig. 34. Effect of pronase (5 mg/ml in Ringer's pH 7.0) on the EOG amplitudes to the odorants. The broken lines indicate the recovery after the washing procedure. (mean \pm SEM of 3 frogs).



PRONASE

After exposure of the olfactory eminence to 5 mg/ml pronase for only 3 minutes in either Ringer's or phosphate buffer (pH 7.0), the solution was withdrawn and EOGs were then recorded (in this particular case the washing procedure did not take place after the enzyme application, whereas in the previous enzyme cases, namely trypsin and semi-alkaline protease, after the incubation procedure the olfactory eminence was washed with Ringer's, and 3 recordings were made to see the effect of the enzyme on the EOG responses to odorants). EOGs to both odorants, n-amyl acetate and 1,8 cineole were reduced with the increase in time. Within 40 minutes of the application of the enzyme, EOGs to n-amyl acetate and 1,8 cineole were reduced by $90 \pm 5\%$ and $83 \pm 3\%$ (mean \pm S.E.M. of 3 frogs) respectively. By washing the mucosa for a period of 10 minutes with Ringer's or phosphate buffer, the eliminated responses to both odorants were fully reversed (Figure 34).

PHOSPHOLIPASE C

Exposure of the epithelium to 1 mg/ml phospholipase C in Ringer's pH 7.0 for one minute irreversibly abolished the EOGs to both odorants, n-amyl acetate and 1,8 cineole. Observations have been made in two frogs and the washing procedure in the Ringer's did not restore the activity.

TRITON X-100

Application of 0.02% triton X-100 in Ringer's pH 7.0 reduced the EOG to n-amyl acetate. This reduction was complete within 120 seconds.

The effect of triton X-100 could not be reversed with the washing procedure.

It has already been mentioned that the olfactory epithelium is covered by a layer of mucus. The thickness of mucus varies from one species to another. It has been observed that its thickness has varied in the same species of frog (Rana temporaria). Graziadei (1971) reported that the thickness of mucus is between 10 μ to 40 μ . In all classes of vertebrates this layer of mucus separates the external environment from the surface of the olfactory receptors. The exact chemical composition of mucus is not known. However, it is reported to consist partially of mucopolysaccharides and glycoproteins (Bannister, 1974). The question is then asked here: do glycoproteins and mucopolysaccharides prevent or delay the effect of proteolytic enzymes reaching the olfactory receptors? If so, a large fraction of mucus could be removed by reducing disulphide bonds in such glycoproteins. A reduction of disulphide bonds in proteins with dithiothreitol (DTT) has been studied (Koningsberg, 1972). Another report indicated that DTT is mucolytic (Lightowler and Lightowler, 1971). In the present study, it was thought that the removal of a large fraction of the olfactory mucus might allow proteolytic enzymes to reach the receptors. DTT was also expected to act as a reagent to reduce the receptor protein sulphides.

The effect of DTT on the responses to odorants elicited from the

frog's olfactory eminence varied among ^{the} 6 frogs studied. This was mainly observed when a low concentration (0.1 mM - 1 mM) of the reagent was used. The effect was changed in the range of $\pm 20\%$ of the original EOG amplitude (100%). The amount of mucus may be involved in this case. Although, when there was a large amount of mucus in the olfactory cavity, the effect of DTT (1 mM, in Ringer's pH 7.0) increased the EOG responses to odorants, n-amyl acetate and 1,8 cineole. This was reversed when there was a small amount of the mucus. However, in every case of the application of a high concentration of the reagent (5 mM - 10 mM), the EOGs to both odorants decreased. In each case, the EOG amplitude to n-amyl acetate was reduced more than that of 1,8 cineole. The reduction of the EOG peak amplitude was seen to be $30\% \pm 11\%$ and that for 1,8 cineole was by about $10\% \pm 6\%$. In one case DTT reduced the EOG response to n-amyl acetate by about 50%, but did not show any effect on the EOG amplitude to 1,8 cineole.

In other experiments, the olfactory mucosa was first treated with DTT (1 mM in Ringer's pH 7.0, for 10 minutes); trypsin (10 mg/ml in Ringer's pH 7.0) was then applied for 5 minutes. Experiments performed on 4 frogs, indicated that the EOG responses to both odorants decreased. The reduction of the EOG amplitude to n-amyl acetate was about $70\% \pm 18\%$, and the reduction of the EOG to 1,8 cineole was by $60\% \pm 15\%$ of the original value. The washing procedure with Ringer's for 10 minutes did not restore the activity of the receptors.

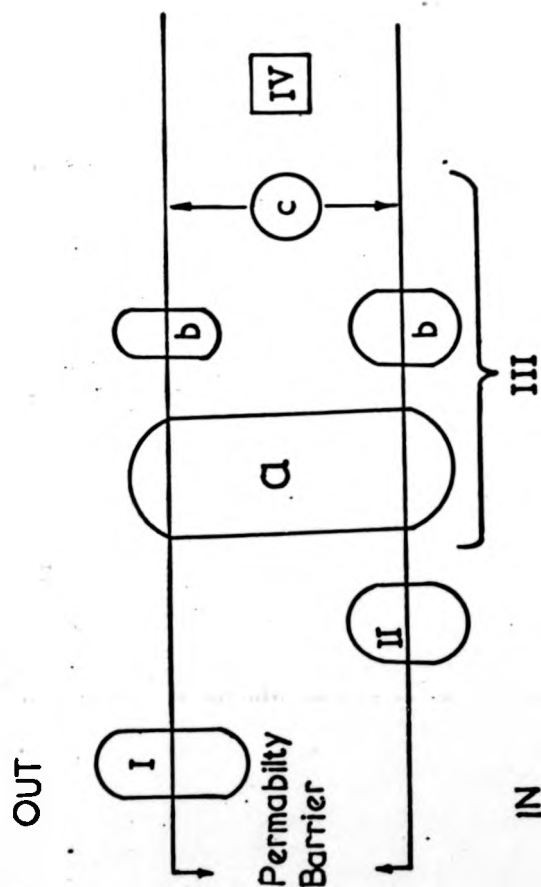


Fig. 35. Four possible topological classes of membrane proteins.
 I- Exterior Surface, II- Interior Surface, III- Transmembrane
 IV- Buried.
 From: Staros and Richards, 1974.

3.6 DISCUSSION

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3.6 DISCUSSION - PART A

Enzymatic Modification Studies

It is clear that there is no strong evidence for the selective removal of any receptors belonging to two different odorants, n-amyl acetate and 1,8 cineole by the incubation of the olfactory eminence with proteolytic enzyme, trypsin. However, the effect observed on the EOG amplitude of n-amyl acetate was somewhat more than that of 1,8 cineole. This might mean that the receptor for n-amyl acetate is more susceptible to the action of proteolytic enzyme than that of 1,8 cineole.

With respect to the dimension normal to the membrane plane, the proteins could be placed in four topologically distinct classes (Staros and Richards, 1974). This is described in Figure 35. Several experimental approaches, including chemical labelling with hydrophilic, small molecule reagents (Steck, 1972) and proteolytic digestion (Triplett and Carraway, 1972), have been used in attempts to determine which of the four classes are actually represented in a particular membrane, i.e. the plasma membrane of human erythrocytes.

In olfaction, the most attractive idea is that there are something like seven to thirty types of receptor proteins (Amoore, 1974). The topological classification of these proteins would again be the same i.e. extrinsic protein, intrinsic protein, transmembrane protein, and buried protein. If extrinsic proteins are receptor proteins for certain

classes of odorants, one would hope that they would be eliminated by the proteolytic enzyme treatment. Proteolysis demands accessibility of a specific group, the susceptible peptide bond, to a macromolecular reagent, the proteolytic enzyme.

Recently it has been demonstrated that the neural responses to sugars were eliminated by proteolytic enzymes (Hiji, 1975). Proteolytic enzymes, such as trypsin (pH 7.0), papain (pH 7.0), neutral (pH 7.0) and acidic proteases applied to the tongue surface of rats for more than an hour did not produce any effect on the neural responses to the four basic stimuli. On the other hand, when pronase (pH 7.0) or semi-alkaline protease (pH 8.0) was applied to the tongue for 20 minutes, the chorda tymphani response to 1 M sucrose was suppressed, whereas responses to the other three taste stimuli were hardly affected. The effects of pronase and semi-alkaline protease found in our work are similar to those of the taste excitation. However, the EOG responses to n-amyl acetate and 1,8 cineole had a similar effect, i.e. one could not be separated from the other, whereas in taste the sugar binding protein was eliminated by proteolytic enzymes.

The recovery of responses to n-amyl acetate and 1,8 cineole is comparable to that of taste responses. After washing away the proteolytic enzyme with Ringer's or phosphate buffer, the effect of enzymes on the EOG responses were reversed (partially in the case of semi-alkaline protease, fully in the case of pronase). It has been suggested (Hiji, 1975) that the recovery of taste responses might be the result of either

the strong repairing action of the protein, or of a rapid turnover mechanism of the receptor protein synthesised in taste cells. This could also explain the recovery of the responses to n-amyl acetate and 1,8 cineole if such an enzyme digestion occurred.

The effect of trypsin on the EOG responses elicited from the DTT treated mucosa may indicate that the trypsin now had accessibility to the receptors, i.e. some part of mucus may have been removed by DTT.

The effect of phospholipase C found in this work is significant. Phospholipase C hydrolyses glycerophosphatides to form a diglyceride and phosphorylcholine. Several experiments strongly suggested the importance of membrane phospholipids in maintaining excitability in excitable cells. For instance, it has been suggested (Rosenberg, et al., 1961) that the action of phospholipase A in blocking the conduction of the squid axon was due to the products of phospholipid splitting, such as lysophosphatides.

Two possible explanations can be given upon the effect of phospholipase C on the EOG responses elicited by n-amyl acetate and 1,8 cineole.

- a) Many odorants are amphipathic molecules; therefore, they can adsorb weakly to phospholipids with very weak association constants (Cherry et al., 1970). According to recent membrane concepts, phospholipids have a fluidity (Singer, 1974). In

this way, one could imagine that the adsorption of an odorant onto a bilayer region of phospholipids near the system (adenylate cyclase) may be responsible for the excitation.

b) Phospholipids may be involved as transducer elements of the olfactory receptor plasma membrane in connecting (linking) the two units of the system.

This system may consist of: a) receptors as discriminators; and b) catalytic unit as an amplifier. These two units may combine after binding of the odorant because of the fluidity of phospholipids in membranes. The disappearance of the EOGs to both odorants after the application of phospholipase C may be the result of phospholipid modification.

The involvement of phospholipids in hormone-receptor interaction has been studied by Birnbaumer *et al.*, (1971). A neutral detergent digitonin caused a loss in the response of ghost adenylate cyclase to all the hormones. It should be noted that the effect of fluoride ion, a stimulator of adenylate cyclase in all tissues, was actually enhanced at low concentrations of the detergents. This finding indicated that fluoride ions activated the enzyme through a separate process, probably by directly affecting the enzyme itself. From these results, membrane lipids might therefore be involved in odour interaction in two ways as explained above, that is, directly or indirectly. A direct mechanism involves interaction between the drug and lipid, and such an interaction might be expected for those odorants, whose actions depend more on their physical properties. Indirect mechanisms involving membrane lipids refer

to changes in the arrangement of the lipids or their interaction with other membrane components which are the consequences of a direct interaction of an odour with a receptor protein.

3.7. RESULTS - PART B

1 - Group Specific Protein Reagents

Application of several group specific protein reagents showed differential effects on the EOG responses evoked by n-amyl acetate and 1,8 cineole. The results presented below are under the name of the reagent concerned. All those reagents applied in liquid phase were dissolved in Ringer's pH 7.0.

4-chloro-7-nitrobenzofurazon (Nbf-Cl)

The work with this chemical modifying reagent has been done in two different phases, vapour phase and liquid phase. In the liquid phase experiments, at concentration of 10^{-4} M in Ringer's (pH 7.0), it has been observed that the EOG recordings to n-amyl acetate and 1,8 cineole were irreversibly abolished. The reagent was in contact with the epithelia for only 2 minutes. The colour of the olfactory eminence had changed from its natural colour, yellow, to dark brown. The washing procedure with Ringer's did not reverse the action of the reagent. After the experiments, the frogs used were kept for observation. On the following day (24 hours later), it was noticed that the frogs which lost their response to odorants after the use of the reagent, started showing responses again. The EOG mV amplitude and waveform characteristics obtained were very similar to those obtained 24 hours earlier prior to the treatment with Nbf-Cl. The epithelia returned again to its normal yellow colour. These experiments were carried out on 2 frogs.

Fig. 36. EOG responses elicited by Nbf-Cl (a) and n-amyl acetate, (b).

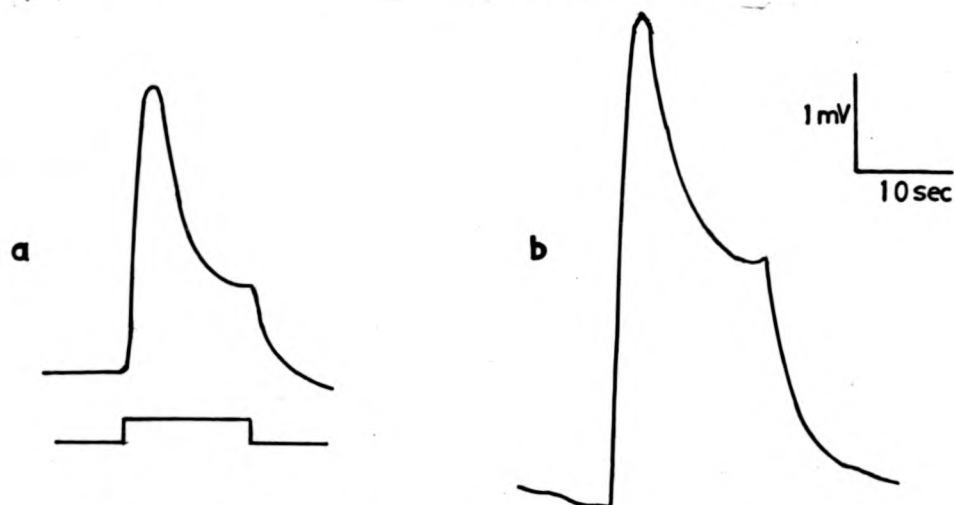
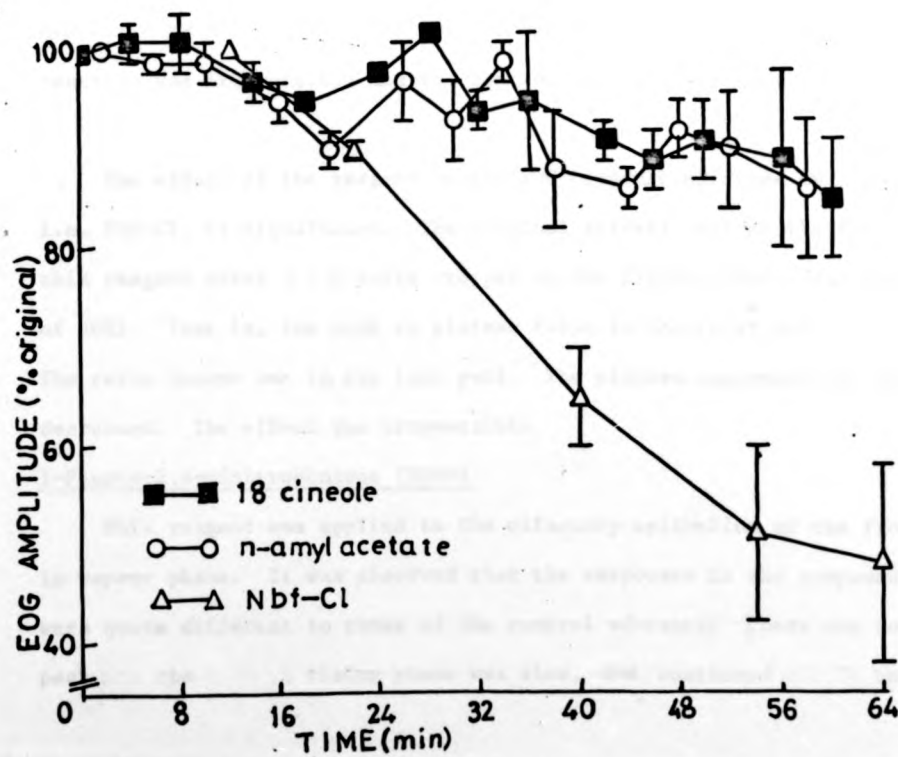


Fig. 37. Effects of Nbf-Cl on the EOG responses elicited by itself. (mean \pm SEM of 3 frogs.)



In vapour phase experiments, Nbf-Cl was puffed onto the olfactory eminence for a short time (10 - 20 sec.) In three frogs the EOG response to this reagent was found to be normal with respect to peak, plateau components and waveform (the rate of rise and the rate of decay of the slow potential, EOG). The EOG to this reagent, i.e. Nbf-Cl, is shown in Figure 36a. It is quite similar to that of n-amyl acetate in respect to the EOG waveform Figure 36b.

In a typical vapour phase experiment, Nbf-Cl was considered as an odorant and was puffed for 10 - 20 seconds with intervals of 2 minutes between each puff. In all three experiments the responses to this reagent were decreased with the increase in the number of puffs. Figure 37 indicates that the EOG peak amplitude to Nbf-Cl was decreased by $52 \pm 11\%$ of the initial value of the compound. The reaction was irreversible and the control odorants were not affected.

The effect of the reagent on the EOG response elicited by itself i.e. Nbf-Cl, is significant. The original (first) peak of the EOG to this reagent after 3 - 6 puffs dropped to the plateau level (the level of 50%). That is, the peak to plateau ratio in the first puff was two. The ratio became one in the last puff. The plateau component was not decreased. The effect was irreversible.

1-Fluoro-2,4-dinitrobenzene (FDNB)

This reagent was applied to the olfactory epithelium of the frog in vapour phase. It was observed that the responses to the compound were quite different to those of the control odorants: there was no peak; the rising phase was slow, and continued until

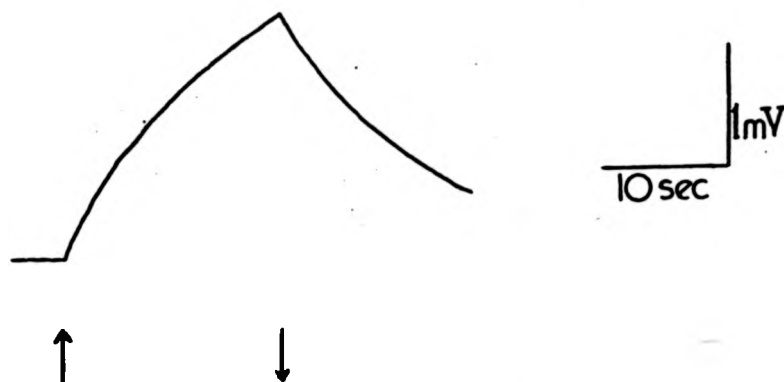


Fig. 38. A response elicited by FDNB from the olfactory mucosa.

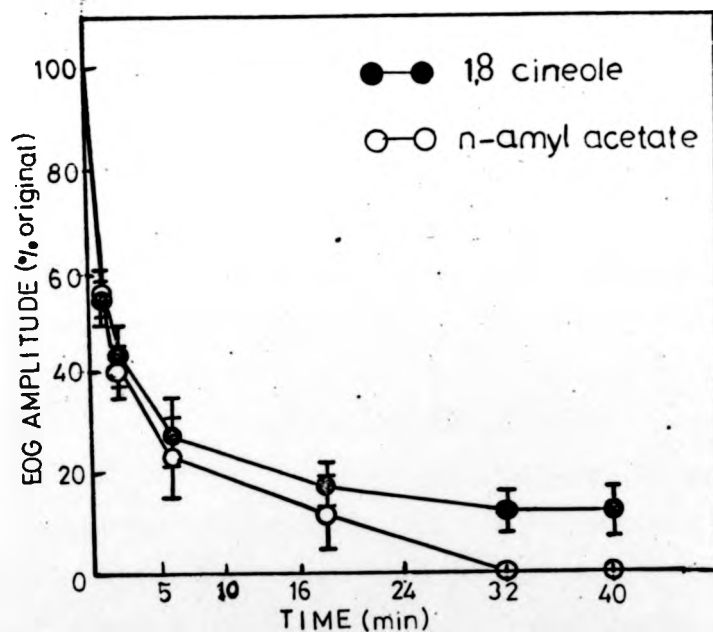


Fig. 39. Time course of the development of the effect of pCMB (in 0.1M glycylglycine buffer, pH 8.0) on the EOG responses to the odorants. (mean \pm SEM of 3 frogs.)

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the end of puffing, again the falling phase was very prolonged (Figure 38).

In the experiments, involving two frogs, this compound was puffed along with one of the control odorants i.e. 1,8 cineole. The compound plus 1,8 cineole was puffed twice for a relatively long period, 5 and 15 minutes respectively. The EOGs to the control odorants were decreased after these two puffs i.e. the EOG amplitude to n-amyl acetate was decreased by $60 \pm 11\%$ and the reduction on the 1,8 cineole amplitude was $22 \pm 7\%$. The effect was irreversible.

P-Chloromercuribenzoic Acid Sodium Salt (pCMB)

The control buffer used to dissolve this reagent was 0.1 M glycylglycine buffer, pH 8.0. Exposure of the epithelium to this buffer increased the amplitude of the EOG to the odorants, n-amyl acetate and 1,8 cineole by about 20 - 30%. After 3 - 4 puffs of the odorants, the EOG responses returned to their original level. The course of the development of the effect of pCMB at 1mM on the EOG amplitudes to odorants is shown in Figure 39 . As is seen from the figure, 10 minutes (total) exposure of the epithelium to this reagent reduces the EOGs to odorants by about 80%; this was completed within 32 minutes with no response to n-amyl acetate, and with $12 \pm 6\%$ to 1,8 cineole. The washing procedure did not cause any recovery of the response.

2,4,6 Trinitrobenzene Sulfonic Acid (TNBS)

Exposure of the epithelium to 10mM TNBS in Ringer's for 5 minutes

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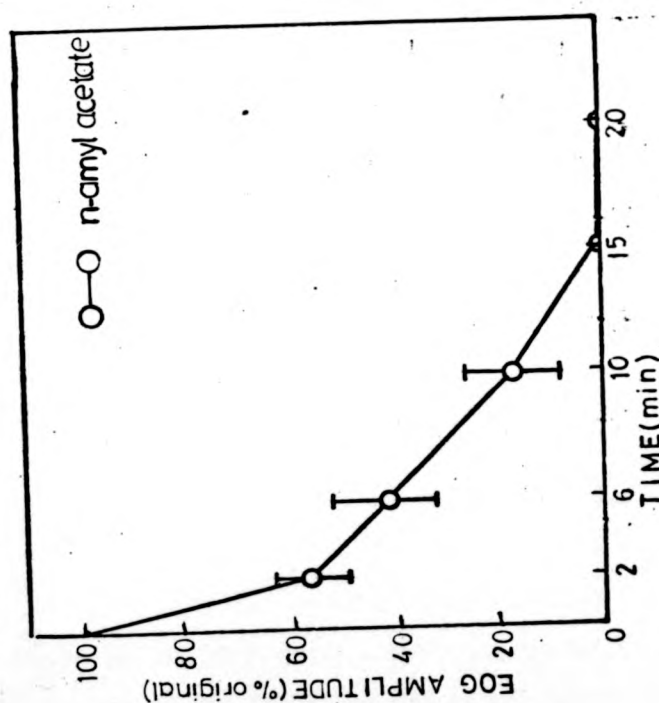
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Effect of pCMB
on the EOG responses

Fig. 40. Time course of the effect of NEM (1mM in Ringer's pH 7.0) on the EOG responses to n-amyl acetate. (mean \pm SEM of 2 frogs.)

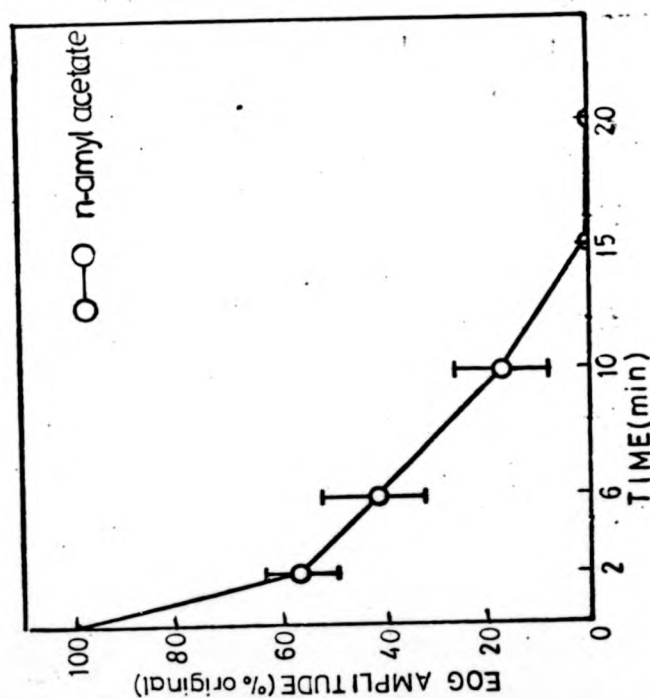


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Fig. 40. Time course of the effect of NEM (1mM in Ringer's pH 7.0) on the EOG responses to n-amyl acetate. (mean \pm SEM of 2 frogs)



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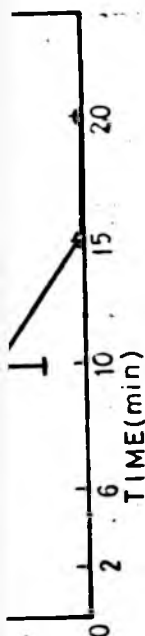
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slightly reduced the EOGs to n-amyl acetate and 1,8 cineole. This reduction of the former was by about $20 \pm 9\%$ (mean \pm S.E.M. of 2 experiments) and of the latter $17 \pm 8\%$ (mean \pm S.E.M.). The application of TNBS on the olfactory eminence was also made after the treatment of the epithelia with a proteolytic enzyme, trypsin (10 mg/ml for 10 minutes).. In a typical experiment, the epithelium was first treated with trypsin and the EOGs to both odorants were decreased by about $30 \pm 8\%$. TNBS (10 mM for five minutes) was then applied on the epithelia. After the removal of the reagents from the mucosa, the EOG responses to both odorants were recorded. The EOGs to both odorants were reduced by about 90% and abolished within 15 minutes after the removal of the reagent, TNBS. There was no recovery for the EOGs to the odorants even though the mucosa had been washed with Ringer's (2 frogs were used for the latter part of the experiment).

N-Ethylmaleimide (NEM)

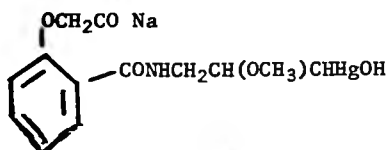
The effects of NEM on the EOG changed when it was applied in liquid and vapour phases. In the vapour phase, where NEM crystals were used, the EOG response to n-amyl acetate was reduced by 50% after a minute of puffing of the compound. The peak to plateau ratio decreased to 1.1 from 2.3, i.e. the effect of NEM was on the peak component of the EOG response. The falling phase of the EOG was also prolonged. In the liquid phase, the effect of 1mM NEM in Ringer's pH 7.0 on the amplitude of the EOG elicited by n-amyl acetate was again found to be time dependent. The full 15 minutes was needed to abolish the EOG to n-amyl acetate (Figure 40). Washing the NEM treated mucosa with Ringer's did not result in a recovery of the EOG response. The similar, but



quicker (7 minutes), effect of 1 mM NEM was found on the responses to ethyl n-butyrate (Getchell and Gesteland, 1972).

Mersalyl

The sulfhydryl agent mersalyl, its structure is shown below, was applied in Ringer's to the frog's olfactory mucosa.



The effects of this reagent on the EOG were found to be concentration dependent. The time course of the development of the effects of four different concentrations (10^{-2} M - 10^{-5} M) of mersalyl in Ringer's (pH 7.0) on the EOG amplitude elicited by n-amyl acetate, is shown in Figure 41. The effects of mersalyl develop quite rapidly. As is shown below, 1 minute exposures of the epithelium to four different concentrations of the agent decreased the EOG responses to the odorant.

| 1 min. application of mersalyl (conc.) | Percentage decreases in the EOG amplitude to n-amyl acetate |
|---|--|
| 10^{-5} M | 30 ± 4 S.E.M. of 2 frogs |
| 10^{-4} M | 54 ± 7 S.E.M. of 4 frogs |
| 10^{-3} M | 64 ± 4 S.E.M. of 3 frogs |
| 10^{-2} M | 83 ± 6 S.E.M. of 2 frogs |

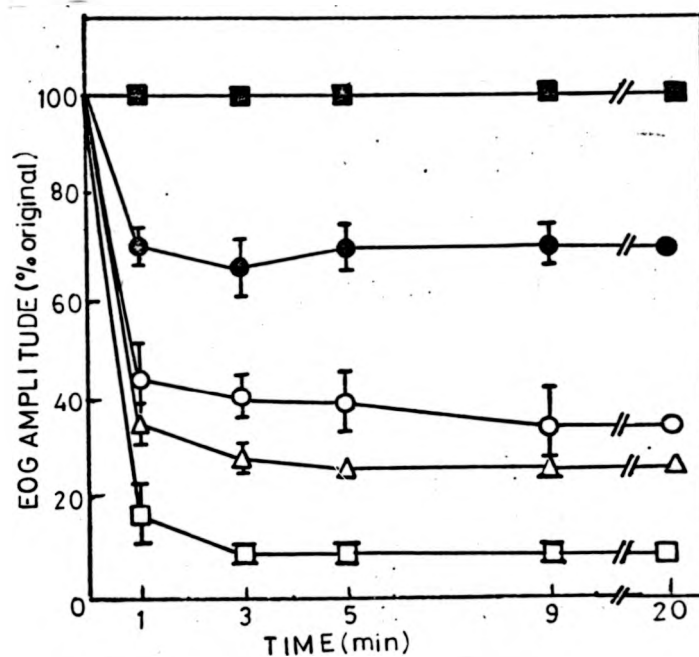
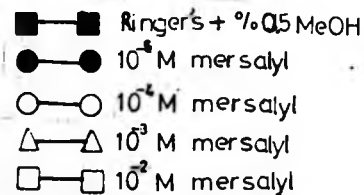


Fig. 41. Time course of the development of the effects of mersalyl (10^{-2} - 10^{-5} M in Ringer's pH 7.0) on the EOG amplitude to n-amyl acetate. (10^{-5} M, mean \pm SEM of 2 frogs; 10^{-6} M, mean \pm SEM of 4 frogs; 10^{-3} M, mean \pm SEM of 3 frogs; 10^{-2} M, mean \pm SEM of 2 frogs).



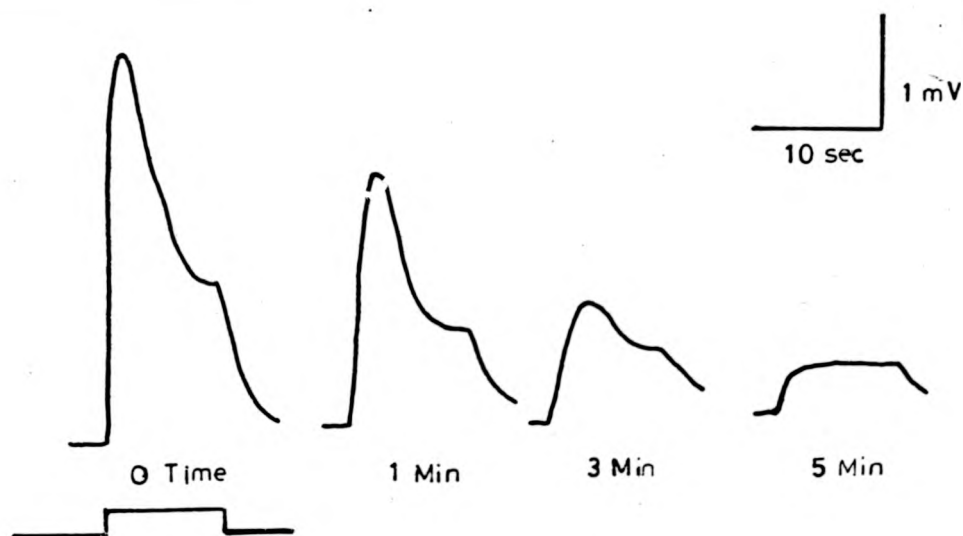


Fig. 42. Time course of the effects of 10^{-6} M mersalyl in Ringer's pH 7.0 on the EOG responses to n-aryl acetate.

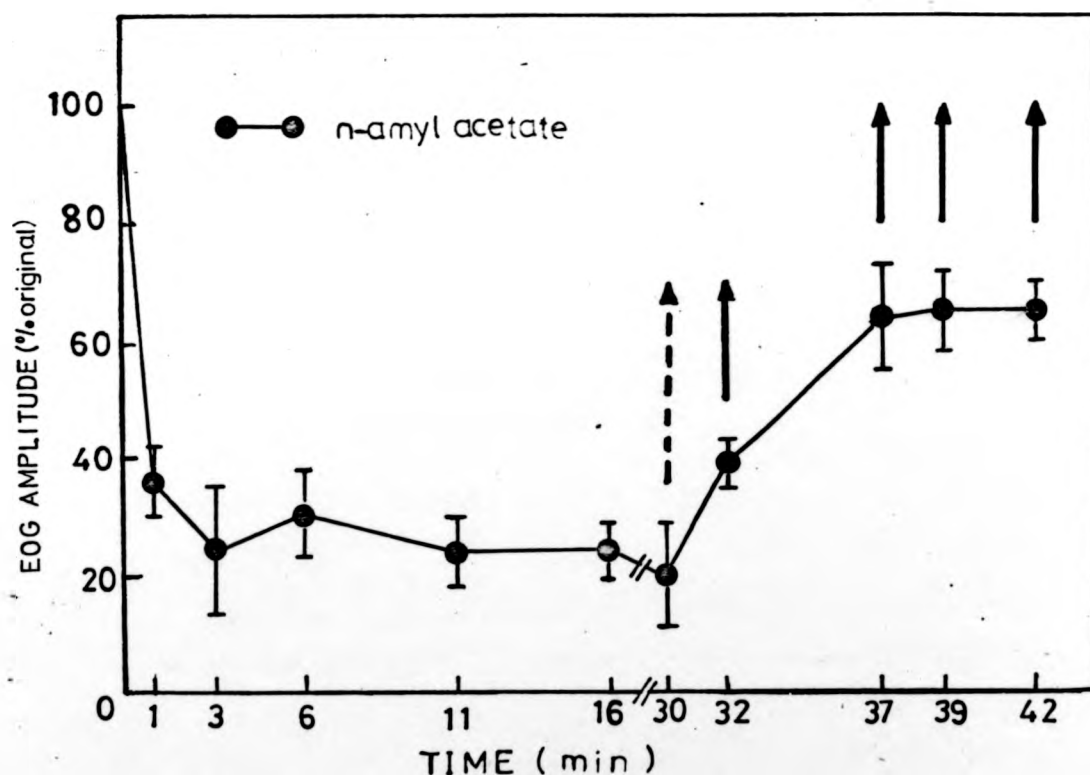
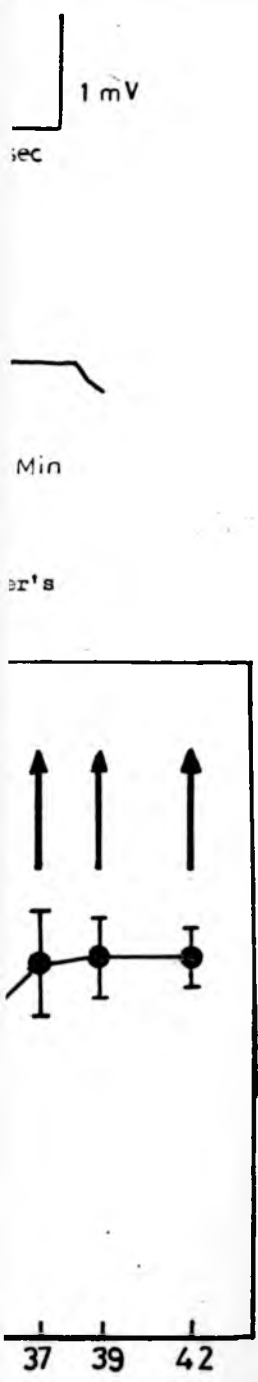


Fig. 43. Time course of the effects of 10^{-6} M mersalyl in Ringer's pH 7.0 on the EOG responses to n-aryl acetate and its subsequent reversibility by DTT (10 mM in Ringer's pH 7.0) (↓; after washing with Ringer's. ↑; after treatment with DTT. Mean \pm SEM of 3 frogs.)

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These reductions, as indicated, are the mean values of a number of experiments which were conducted on different frogs. The EOG was not totally abolished at the highest (10^{-2} M) mersalyl concentration used. The effects of mersalyl at all concentrations were not reversed by washing the mucosa with Ringer's for the same period of time as it was exposed to the reagent.

Treatment of the epithelium with mersalyl had pronounced effects on the waveform of the EOG. The normal EOG elicited by n-amyl acetate consists of two components, a peak and a plateau (Figure 42). There are no other components involved in the shape of the EOG. Exposure of the epithelium to mersalyl caused equal decreases in both peak and plateau amplitudes. The peak to plateau ratio was initially unchanged. However, with the increase in the application time of the reagent, the peak disappeared, and the plateau was decreased. Representative EOGs from one experiment of this type, with the use of 10^{-4} M mersalyl in Ringer's pH 7.0 are presented in Figure 42.

The inhibition brought about by mersalyl was also observed on the EOGs to 1,8 cineole, butyric acid, phenylacetaldehyde, etc. (see below). In every case the effect was immediate and was not reversed by the washing procedure with Ringer's. However, the effect of mersalyl could partially be reversed by DTT. Figure 43 shows that the full 16 minutes application of 10^{-3} M mersalyl decreased the EOG to n-amyl acetate by $76\% \pm 5\%$. The washing procedure with Ringer's did not reverse the effect of the reagent, in fact 15 minutes washing of the

mucosa with Ringer's resulted in a slight decrease in the response, from 76% to 80%. If the mucosa was then exposed to DTT, 10 mM in Ringer's for 2 minutes, the response to n-amyl acetate was increased by about $20\% \pm 4\%$. Further applications of DTT increased the responses by another 25%, bringing the responses up to $65\% \pm 5\%$ of the control value (before the effect of mersalyl was observed).

It was noticed that the responses to the odorants were abolished an hour after the application of mersalyl. Two - three hours after the application of the reagent, EOG recordings could not be obtained.

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)

Treatment of the epithelium with this reagent, at a concentration of 1 mM in Ringer's, slightly (5% - 10% of the control) increased the EOGs to n-amyl acetate and 1,8 cineole. After the exposure of the epithelium to DTNB, it was noticed that the resting potential (the base line) had changed in the negative direction by tens of millivolts. This is exactly the opposite to the effect of DTT found on the resting potential, where the effect changed the resting potential in the positive direction by tens of millivolts. It has been reported that the reducing agent DTT, when applied to the innervated membrane of the electroplax of Electrophorus electricus, inhibited the depolarising response caused by ACh or carbamyl choline. This inhibition could be reversed by subsequent application of an oxidising agent such as DTNB. However, when a sulphhydryl alkylating agent such as NEM was applied to the preparation that had been treated with DTT, DTNB could no longer reverse the inhibition caused by DTT. Therefore, it was concluded that

a disulfide group appeared to be related to the ACh receptor (Karlin and Bartels, 1966).

Treatment of the olfactory mucosa with DTT at high concentrations (5 - 10 mM), as was mentioned earlier, reduced the EOGs to n-amyl acetate and 1,8 cineole. This reduction varied from one frog to another, depending on the amount of mucus present on the olfactory epithelium. However, this reduction was around $30\% \pm 9\%$ (mean \pm S.E.M. of 4 experiments) of the control amplitude. The decrease brought about by DTT (10 mM in Ringer's, pH 7.0) was reversed by DTNB and even increased the response by about 20% - 30% of the control value. The increase of the EOG responses to the odorants was also observed with this reagent, DTNB, if the mucosa was first exposed to TNBS which reduced the responses by about $20\% \pm 11\%$ (mean \pm S.E.M. of 3 experiments). That is, the effect of TNBS was reversed by DTNB.

In other experiments, where the mucosa was first treated with DTT, and NEM was then applied, the responses were abolished; DTNB could not reverse the inhibition. This was also seen when NEM was applied first, where NEM brought about a complete inhibition in the responses; this could not be reversed by DTNB. In another type of experiment, where the mucosa was first exposed to TNBS, the responses to both odorants were reduced, DTNB reversed the activity to, or above the control value; if the epithelium was then washed with Ringer's the responses were completely abolished. The effect brought about by the washing procedure (Ringer's) on the TNBS and DTNB treated mucosa was reversed to, and increased above the control value by DTNB.

2 - PROTECTION EXPERIMENTS

Rationale and Preliminary Experiments

A promising approach to the receptor properties was conducted in an experiment associating electrophysiological and biochemical techniques (Getchell and Gesteland, 1972). It was reported that exposing the nasal cavity to NEM, which reacts with sulfhydryl groups of proteins, irreversibly blocked the EOG responses to odour stimuli in the frog. However, receptors could be specifically protected by an odorous substance saturating the receptor before and during the presence of NEM. This "protection" of the response to the protecting odorant itself also extended to the responses of closely related compounds. These findings gave a direct experimental basis to the view that odorous stimuli interact with relatively specific sites on the plasma membrane of the receptor cell.

Results in the previous section (1) of this chapter have shown the modification of receptors as reflected in EOG responses to odorant compounds, which were brought about by group specific protein reagents. To establish that the modification was a genuine receptor event, protection experiments were carried out in this section. As in the modification of enzyme active sites, the expectation here would be that receptor site labelling would be prevented by adsorption of odorants at the receptor sites. The technique used here for the protection experiments is somewhat similar to that of Getchell and Gesteland (1972)

with respect to sequential applications of firstly odorant in solution, then odorant plus group specific reagent, and finally odorant in solution. This sequence had to be followed because the protection by any other means could not be achieved (see the following section). I have used a different odorant, group specific reagent, and exposure time to those used by Getchell and Gesteland (1972).

Mersalyl was chosen as the group specific reagent for the protection experiments for the following reasons:

- a) it is not a penetrant reagent (unlike Getchell and Gesteland's group specific reagent, NEM which is penetrant - Vignais and Vignais, 1973), and therefore can only act at the surface of the membrane,
- b) its effects are irreversible and short exposures of the olfactory eminence to low concentrations (10^{-4} M) of this reagent have large effects on the EOG amplitude and waveform,
- c) it is water soluble,
- d) it reacts specifically with free and reactive -SH groups, its use in our work would be useful and give further evidence in elucidating the possible involvement of -SH group in the odorant-receptor binding.

The affinity of the sulfhydryl reagents for sulfhydryl groups which are present on the protein is quite high (low K_D), and the possible interaction expected between the two is the formation of a covalent bond which is irreversible to washing. It is unlikely that the covalent interaction is involved in the odour-receptor binding

phenomenon. It has recently been reported (Dodd, 1976) that such covalent interactions would result in two undesirable features:

- 1) the molecule would have a reactive functional group which would make it a trigeminal stimulant;
- 2) the molecule would be a substrate for an enzyme involving a covalently bound intermediate.

In olfaction, because of a wide range of stimulants, odorants may act as regulatory molecules rather than as substrates. The association of an odorant and its receptor site, therefore, involves much weaker forms of bonding (van der Waals, repulsion, hydrophobic, electrostatic and hydrogen bonds). Since the specificity of a binding site for a ligand is reflected in the binding constant (K_D), for that ligand with its receptor, with the high K_D value the specificity of the odorants for their receptors will be low, (K_D may be in the μM region for many classes of odorants). As a result of this odours will show a much lower affinity (high K_D) for the receptor sites than will sulphhydryl reagents.

n-Amyl acetate was chosen as the stimulus, to protect its receptor against mersalyl for the following reasons:

- a) n-Amyl acetate elicits a large EOG (3 - 6 mV) compared to other odorants used, and its waveform is characterised by a high rate of rise compared to its rate of decline. Because large amplitude EOGs to n-amyl acetate are obtained, it can be assumed that the number of receptors is large and the affinity of this odorant for the receptor sites is relatively low. It may be, therefore, that the lower the specificity, the higher the EOG will be (high

K_D). For instance, the musks are relatively high molecular weight odorants and frequently have a number of specificity determinants on the molecule. Many of the steroid musks are related to progesterone (it binds to its receptor, rat liver cytosol with a K_D value of 0.023 μ M (Dodd, 1976)) and may exhibit similar tight binding; it can therefore be assumed the musks bind more tightly than many odorants. Results have indicated that the musks - musk ketone, musk ambrette and nitro musks - elicited very small EOGs in terms of millivolt amplitude value (0.2 - 0.8 mV). Their EOG waveform is also different from that of n-amyl acetate. The rate of rise and decay is twice as slow as that of n-amyl acetate. In most cases a peak was absent in the EOGs. Despite the fact that the vapour pressure for the musks was changed (increased), there were no increases in the EOGs. Therefore, one could assume that the musks bind specifically to their receptors with the low K_D value and elicit small EOGs. An alternative explanation for the small musk EOGs is that frogs may lack musk receptors. This has been backed by the fact that one or two frogs out of 50 elicited good EOGs (2 - 3 mV) to the musks. This may indicate that the receptors for the musks are special ones which may be genetically determined.

- b) The effect of mersalyl on the EOG elicited by n-amyl acetate was well-characterised.
- c) Exposing the mucosa to 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH, pH 7.0 for 12 minutes caused a reduction of $39\% \pm 3\%$ (mean

\pm S.E.M. of 5 experiments) of the control EOG value. The effect was reversible. The EOG amplitude recovered spontaneously with time, and washing the mucosa with Ringer's speeded up the recovery process so that it was completed within 38 minutes. (Figure 44).

- d) No reaction would be expected to occur between mersalyl and n-amyl acetate in solution.

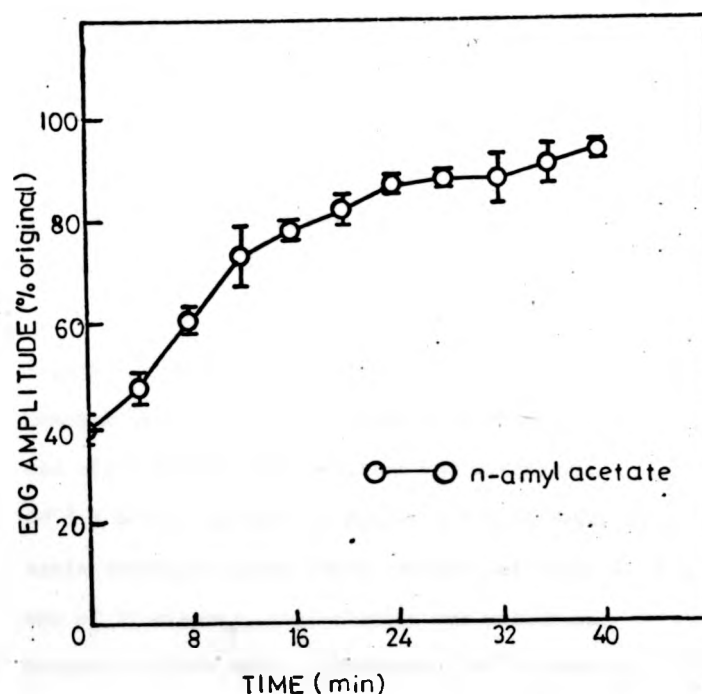


Fig. 44. Recovery of the EOG responses to n- amyl acetate after the treatment of the olfactory mucosa with 10^{-3} M n-amyl acetate in Ringer's-%0.5 MeOH. (mean \pm SEM of 5 experiments.)

Demonstration of Protection

The protection experiments were carried out according to the following pattern:

1. An EOG to n-amyl acetate was recorded from the untreated mucosa to see whether the frog is responding normally.
2. The nasal cavity was filled with Ringer's + 0.5% MeOH; the solution was withdrawn and replaced after two, five and eight minutes. At the end of ten minutes, the solution was withdrawn.
3. Three EOGs to n-amyl acetate were recorded. The average of the amplitudes to these three EOGs was the control amplitude (100%).
4. The mucosa was exposed to one of the following sequences of treatments:
 - A) Ringer's + 0.5% MeOH for 12 minutes. The pattern of solution changes were carried out after 4 minutes, 8 minutes and at the end of 12 minutes the solution was withdrawn.
 - B) 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH for 12 minutes, again solution changes were carried out after 4, 8 and at the end of 12 minutes, the solution was withdrawn.
 - C) Ringer's + 0.5% MeOH, 4 minutes; 10^{-4} M mersalyl, 4 minutes; Ringer's + 0.5% MeOH, 4 minutes.
 - D) 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH, 4 minutes; 10^{-3} M n-amyl acetate + 10^{-4} M mersalyl in Ringer's + 0.5% MeOH, 4 minutes; 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH, 4 minutes.

5. The nasal cavity was filled with Ringer's + 0.5% MeOH as in Step 2.
6. EOGs to n-amyl acetate were elicited and recorded every 3 minutes for the next 39 minutes.

When the epithelium was exposed to 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH for 12 minutes as above (Step 4B), and then to Ringer's + 0.5% MeOH for 10 minutes as in Step 5, the EOG amplitude was reduced by about $39\% \pm 3$ (mean \pm S.E.M. of 5 experiments). The response amplitude recovered within 40 minutes as shown in Figure 44.

When the mucosa was treated with Ringer's + 0.5% MeOH for 4 minutes, 10^{-4} M mersalyl in Ringer's 0.5% MeOH again for 4 minutes (Step 4C), and the repeat of Step 5, the amplitude was reduced by about $66\% \pm 6$ (mean \pm S.E.M. of 18 experiments) and the EOG recordings indicated that there was no recovery as shown in Figure 45. However, when 10^{-3} M n-amyl acetate was present before, during and after exposure to mersalyl (Step 4D), washing the mucosa with Ringer's + 0.5% MeOH caused the EOG amplitude to recover to $91\% \pm 4$ (mean \pm S.E.M. of 24 experiments) of its original value (Figure 45). Thus the odorant was able to protect the receptor sites from the effects of mersalyl.

If the mucosa in which the protection had occurred was treated for 2 minutes with the same solution of 10^{-3} M n-amyl acetate + 10^{-4} M mersalyl in Ringer's + 0.5% MeOH that had been used on it previously

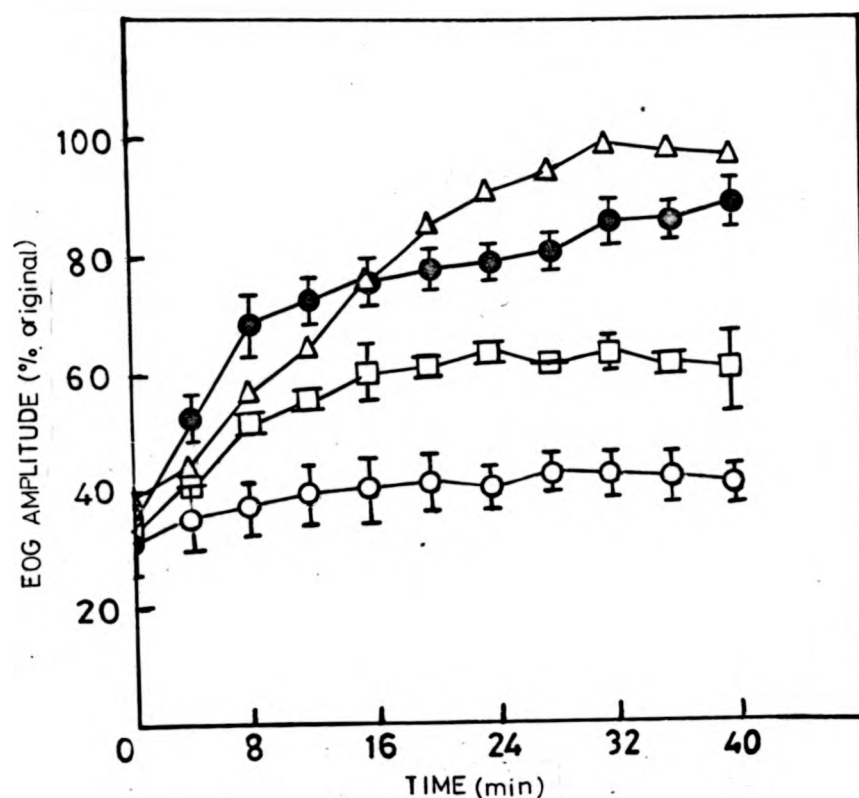


Fig. 45. Protection of the EOG responses to n-amyl acetate from the effects of mersalyl by n-amyl acetate in solution.(see text)

- 10^{-4} mersalyl alone (mean \pm S.E.M. of 18 frogs)
- 10^{-4} mersalyl + 10^{-4} n-amyl acetate (mean \pm S.E.M. of 4 frogs)
- 10^{-4} mersalyl + 10^{-3} n-amyl acetate (mean \pm S.E.M. of 24 frogs)
- △—△ 10^{-4} mersalyl + 10^{-2} n-amyl acetate (1 frog)

(but without prior and subsequent exposure to the odorant alone), the amplitude of the EOG to a puff of n-amyl acetate was reduced by about 80 - 90%. The washing procedure with Ringer's + 0.5% MeOH afterwards failed to cause any recovery in amplitude. This indicates, as has been discussed above, that the receptors to n-amyl acetate have to be saturated ^{against} the effect of mersalyl by the prior treatment of the mucosa with 10^{-3} M n-amyl acetate in solution.

n-Amyl acetate binding to its receptor with high K_D value, (low affinity), cannot compete with mersalyl molecules which with low K_D value, (high affinity). Mersalyl molecules bind covalently to the receptor and EOG responses to n-amyl acetate show no sign of recovery.

Protection experiments were also carried out by changing the odorant concentration in solution and keeping the reagent concentration constant. Results have shown that with increases in the n-amyl acetate concentration in solution, a better protection of the receptors was achieved.

In all protection experiments described above the time period for the application of solutions were not changed. In some protection experiments, however, the exposure time for solutions were changed. Increases and decreases in the exposure time for n-amyl acetate in solution before and after the application of mersalyl did not change the results obtained above. Neither did the increase in the exposure time to mersalyl alter the results. The presence of an odour in solution before, during and after the

(but without prior and subsequent exposure to the odorant alone), the amplitude of the EOG to a puff of n-amyl acetate was reduced by about 80 - 90%. The washing procedure with Ringer's + 0.5% MeOH afterwards failed to cause any recovery in amplitude. This indicates, as has been discussed above, that the receptors to n-amyl acetate have to be saturated ^{against} the effect of mersalyl by the prior treatment of the mucosa with 10^{-3} M n-amyl acetate in solution.

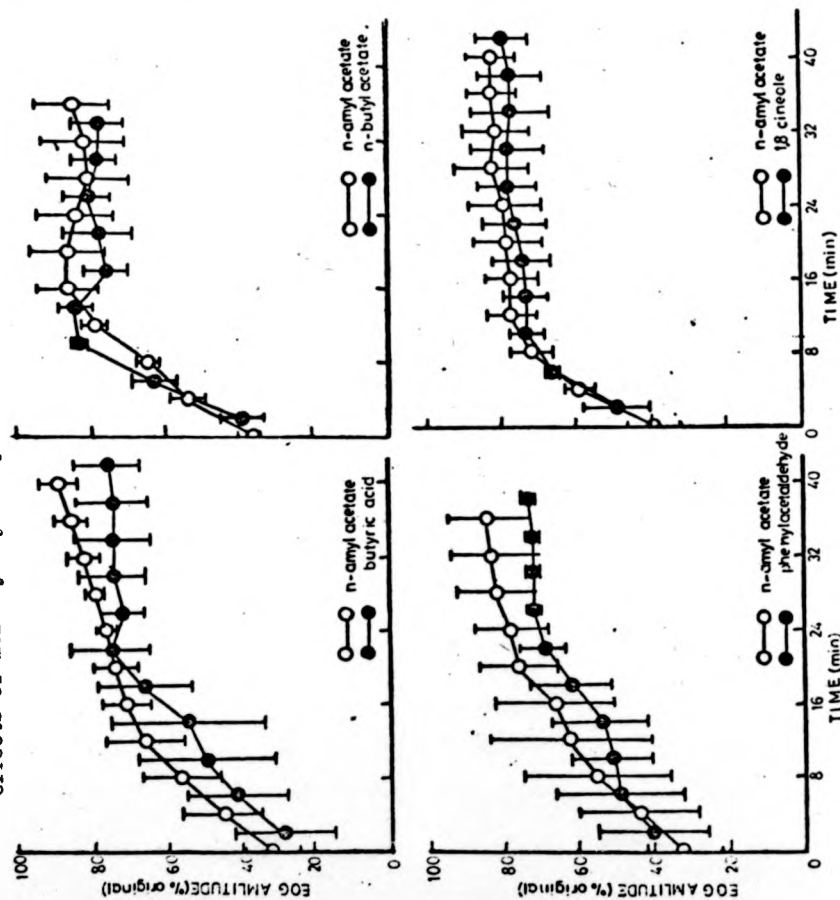
n-Amyl acetate binding to its receptor with high K_D value, (low affinity), cannot compete with mersalyl molecules which with low K_D value, (high affinity). Mersalyl molecules bind covalently to the receptor and EOG responses to n-amyl acetate show no sign of recovery.

Protection experiments were also carried out by changing the odorant concentration in solution and keeping the reagent concentration constant. Results have shown that with increases in the n-amyl acetate concentration in solution, a better protection of the receptors was achieved.

In all protection experiments described above the time period for the application of solutions were not changed. In some protection experiments, however, the exposure time for solutions were changed. Increases and decreases in the exposure time for n-amyl acetate in solution before and after the application of mersalyl did not change the results obtained above. Neither did the increase in the exposure time to mersalyl alter the results. The presence of an odour in solution before, during and after the

application of the reagents was essential for protection,
regardless of the exposure time of solutions.

Fig. 46. Protection of the EOG responses to butyric acid, n-butyl acetate, phenyl acetaldehyde and 1,8 cineole from the effects of mersalyl by n-amyl acetate in solution. (see text)



In the sites which by n-amyl a question as responses t

In thi different vapour phase epithelium acetate in 0.5% MeOH. respond to primary cl protection ambrette, n-butylace n-amyl ace from the e for these can be see 70% - 80%

3 SPECIFICITY OF PROTECTION

In the previous part of the work, it was shown that the receptor sites which interact with n-amyl acetate in vapour phase were protected by n-amyl acetate in solution against the effect of mersalyl. The question asked here is: could n-amyl acetate also protect the responses to other odorants?

In this part of the work a group of odorants with structures different to that of n-amyl acetate were used as stimuli in the vapour phase, and the protection experiments on the olfactory epithelium described above were repeated, using 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH and 10^{-4} M mersalyl in Ringer's + 0.5% MeOH. Difficulties arose from the fact that the frogs did not respond to some of the odorants which were picked from each of the primary classes of Amoore (1971). Therefore, the specificity of the protection were not carried out for the odorants: musk ketone, musk ambrette, menthol, phenylethanol etc. The results for butyric acid, n-butylacetate, phenylacetaldehyde and 1,8 cineole have shown that n-amyl acetate is able to protect the receptor site for these odorants from the effects of mersalyl (Figure 46). The percentage recovery for these odorants after 34 minutes is shown in the table below. It can be seen that the EOGs to these odorants recovered by around 70% - 80% of their control responses.

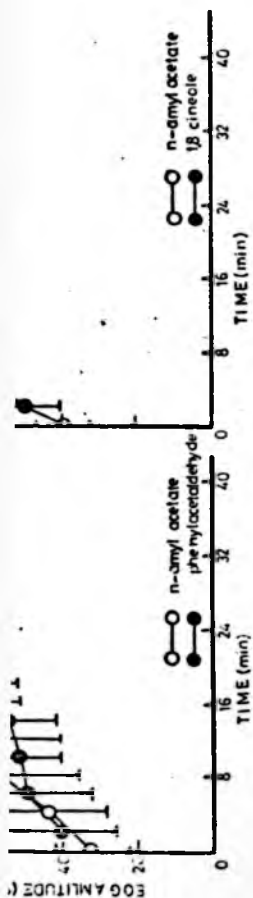
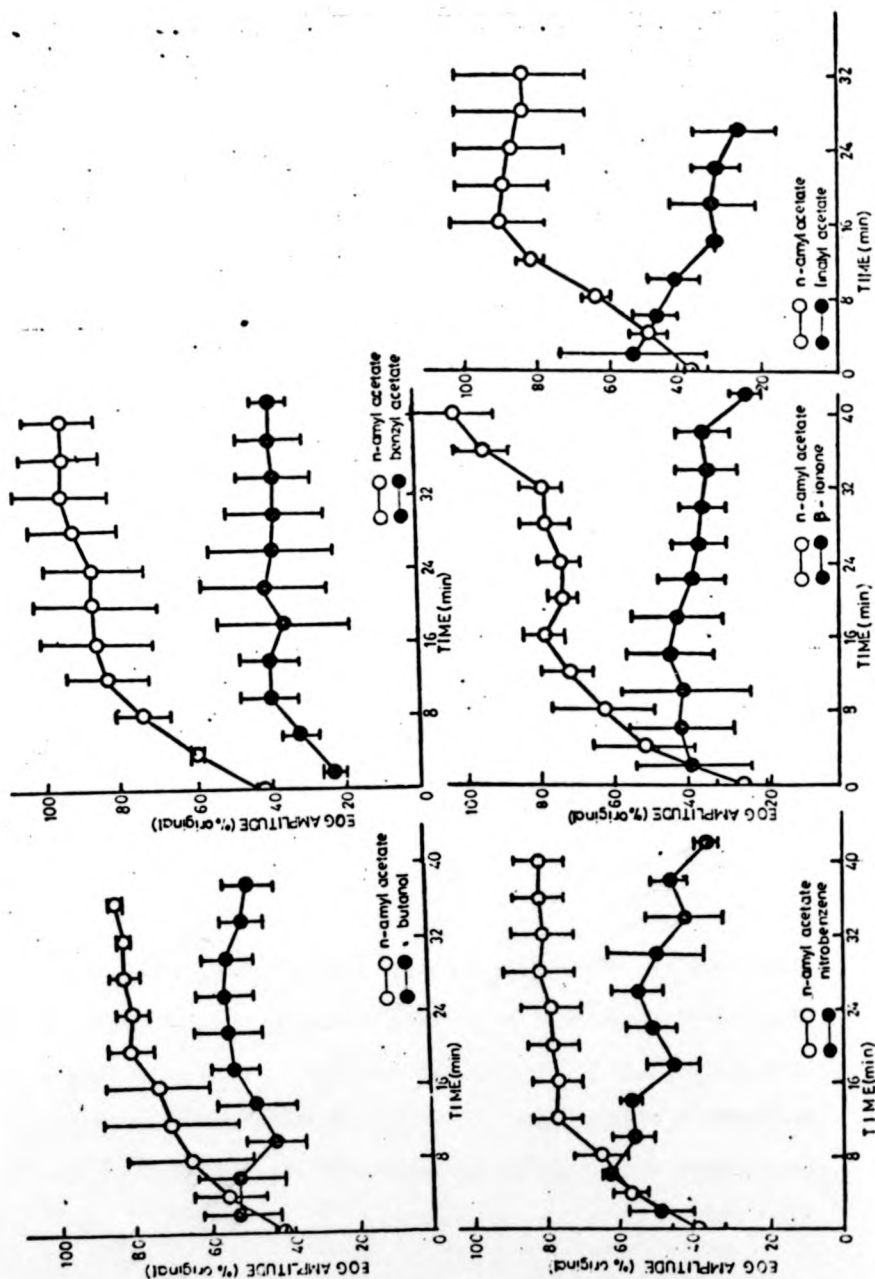


Fig. 47. Lack of the protection of the EOG responses to butanol, benzyl acetate, nitrobenzene, β -ionone and linalyl acetate from the effects of mersalyl by n-amyl acetate. (see text)

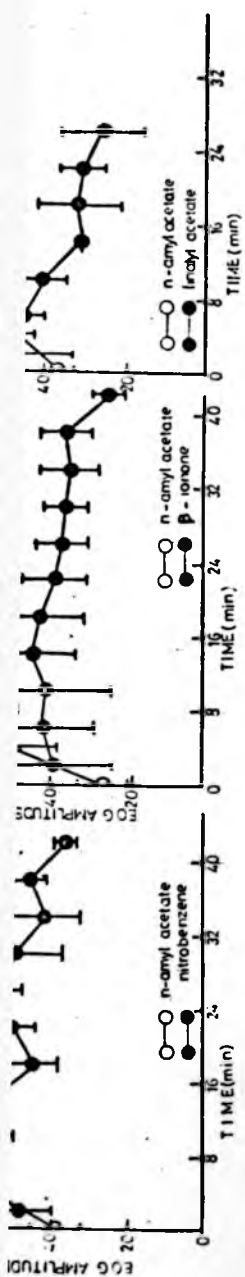


Odor
Butyric acid
n-Butyl acetate
Phenylacetate
1,8 cineole

The EOG response to
nitrobenzene
after the peak
mean recovery
indicating
by n-amyl acetate

Odor
Butanol
benzyl acetate
Nitrobenzene
 β -ionone
Linalyl acetate
(at 24 min)

As is shown, the
specificity of the
olfactory response



| Odour | % Protection after 34 minutes | No. of frogs |
|--------------------|---------------------------------|--------------|
| Butyric acid | 74 \pm 10 (mean \pm S.E.M.) | 3 |
| n-Butyl acetate | 78 \pm 7 " | 3 |
| Phenylacetaldehyde | 72 \pm 2 " | 2 |
| 1,8 cineole | 77 \pm 11 " | 3 |

The EOG responses to the odorants; butanol, benzyl acetate, nitrobenzene β -ionone and linalyl acetate did not show any recovery after the protection experiments. The table below shows that the mean recovery value of the responses are between 50% and 28% indicating the EOG responses to these odorants were not protected by n-amyl acetate.

| Odour | % Protection after 34 minutes | No. of frogs |
|---------------------------------|--------------------------------|--------------|
| Butanol | 50 \pm 6 (mean \pm S.E.M.) | 2 |
| benzyl acetate | 38 \pm 10 " | 2 |
| Nitrobenzene | 42 \pm 10 " | 3 |
| β -ionone | 35 \pm 8 " | 4 |
| Linalyl acetate (at 24 minutes) | 28 \pm 11 " | 2 |

As is shown in Figure 47 in every experiment concerning the specificity of the protection, n-amyl acetate was also puffed to the olfactory mucosa. This provided further evidence that the protection

experiments were genuine as to the elimination or the protection of the particular odorant responses concerned. Nevertheless, in every case n-amyl acetate protected its receptors against the effect of mersalyl.

Protection of the EOGs to butyric acid, n-butyl acetate, phenylacetaldehyde and 1,8 cineole from the effects of mersalyl by n-amyl acetate in solution.

The experiments on each odorant concerned were carried out according to the following pattern:-

The olfactory mucosa was exposed to Ringer's + 0.5% MeOH for 10 minutes; 3 EOGs were elicited by each of the odorants after withdrawal of the Ringer's + 0.5% MeOH, the average of each 3 EOGs was the control response (100%) for that particular odorant. The mucosa was then subjected to one of the following procedures:

1. 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH for 12 minutes, solution changes were carried out after 4 and 8 minutes, and then at the end of 12 minutes, the solution was withdrawn. The mucosa was then washed with Ringer's + 0.5% MeOH for 10 minutes. EOGs were elicited by each odorant for the next 40 minutes. The peak amplitudes of these EOGs are represented by the curves on each of the graphs.

The graphs relating to the effect of this solution, i.e. n-amyl acetate (10^{-3} M) indicated that the EOG responses to the odorants, butyric acid, n-butyl acetate, phenylacetaldehyde and 1,8 cineole recovered almost to their original value within 40 minutes (Figure 48).

2. Ringer's + 0.5% MeOH, 4 minutes; 10^{-4} M mersalyl, 4 minutes; Ringer's + 0.5% MeOH, 4 minutes. The mucosa was then washed with Ringer's + 0.5% MeOH for 10 minutes. EOGs were evoked by each odorant

Fig. 48. Recovery of the EOG responses to butyric acid, n-butyl acetate, phenyl acetaldehyde and 1,8 cineole from the effects of 10^{-3} M n-amyl acetate in solution. (see text)

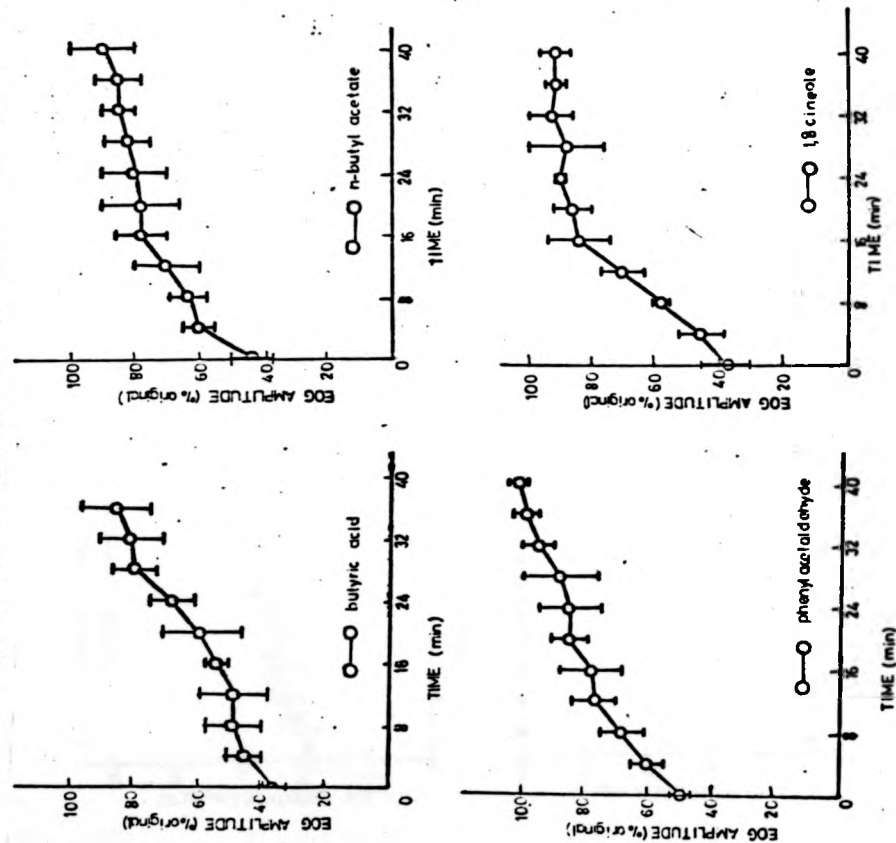
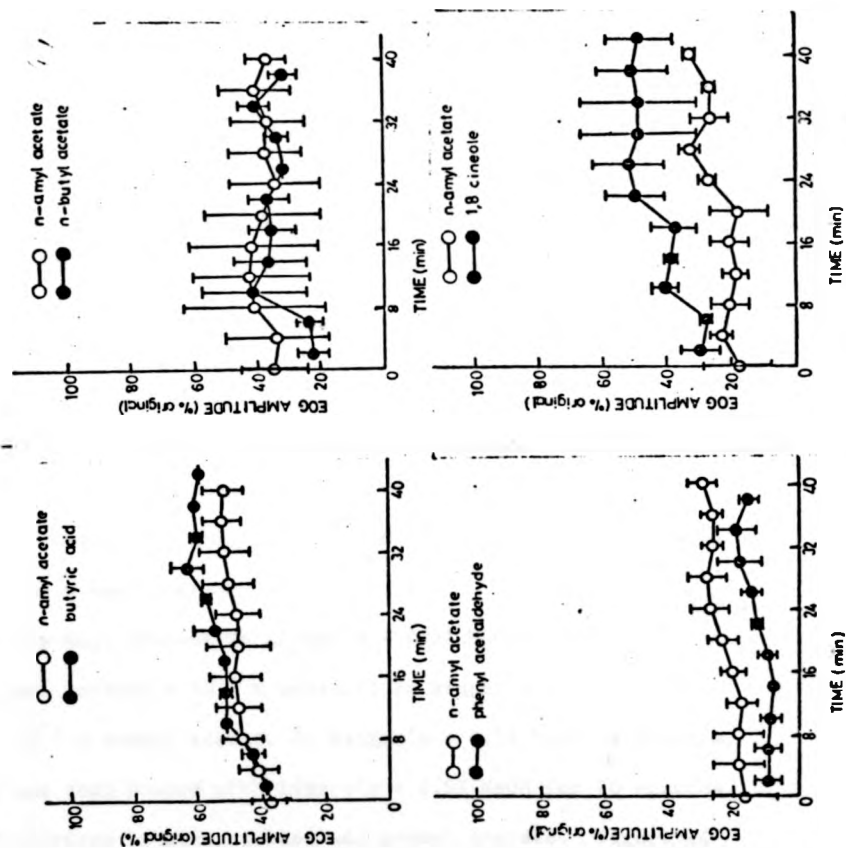


Fig. 49. Effect of 10^{-6} M mersalyl on the ECG responses to butyric acid, n-butyl acetate, phenyl acetaldehyde and 1,8 cineole.



and n-amyl acetate for the next 40 minutes. The EOG responses to the odorants as well as to n-amyl acetate did not show any recovery from the effect of mersalyl alone (Figure 49). At the end of 38 minutes of recording the EOG responses obtained are described in the table below.

| Odour | % Recovery after 38 minutes | No. of frogs |
|--------------------|----------------------------------|--------------|
| Butyric acid | 60 \pm 0.0 (mean \pm S.E.M.) | 2 |
| n-Butyl acetate | 31 \pm 4 " | 2 |
| Phenylacetaldehyde | 15 \pm 3 " | 2 |
| 1,8 cineole | 32 \pm 1 " | 2 |

This indicates that the treatment with just 10^{-4} M mersalyl caused an irreversible inhibition on the responses to the odorants as well as to n-amyl acetate.

3. 10^{-3} M n-Amyl acetate in Ringer's + 0.5% MeOH, 4 minutes;
 10^{-3} M n-amyl acetate + 10^{-4} M mersalyl in Ringer's + 0.5% MeOH, 4 minutes; 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH, 4 minutes. The mucosa was then washed with Ringer's + 0.5% MeOH for 10 minutes. EOGs were elicited by each odorant and n-amyl acetate. Figure 46 shows that the responses to these odorants as well as to n-amyl acetate recovered with the increase in time. This implies that the recovery depends on the presence of n-amyl acetate in solution before, during and after the treatment of the mucosa with mersalyl. Therefore, with n-amyl acetate in solution, the receptors to the odorants butyric acid, n-butyl acetate, phenylacetaldehyde and 1,8 cineole

were protected (Figure 46). But without such a protector i.e. n-amyl acetate, the receptors to the aforementioned odorants were not protected (Figure 49).

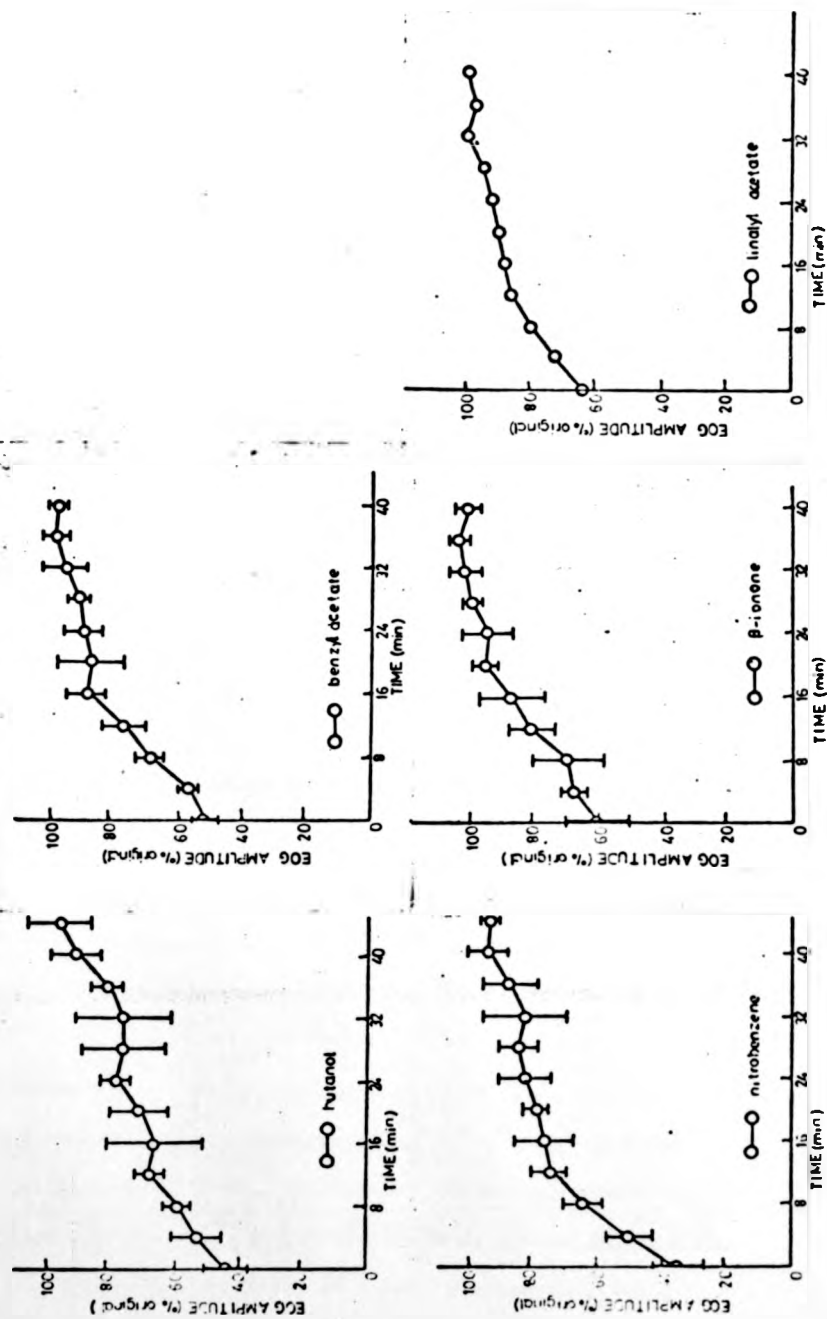
Lack of Protection of the EOGs to butanol, benzylacetate, nitrobenzene, β -ionone and linalyl acetate from the effects of mersalyl by n-amyl acetate in solution.

The experiments were carried out for each odorant concerned according to the following pattern:

The olfactory mucosa was exposed to Ringer's + 0.5% MeOH for 10 minutes; 3 EOGs were elicited by each of the odorants after withdrawal of the Ringer's + 0.5% MeOH, the average of the EOGs was the control response (100%) for that particular odorant. The mucosa was then treated with one of the following solutions.

1. 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH for 12 minutes; solution changes were carried out after 4 and 8 minutes and, at the end of 12 minutes, the solution was withdrawn. The mucosa was then washed with Ringer's + 0.5% MeOH for 10 minutes. EOGs were elicited by each odorant for the next 40 minutes. The results showed that the treatment of the mucosa with n-amyl acetate in solution initially decreased the responses to the odorants. But the recovery was observed in the responses as the time period progressed. The recovery in the responses to the odorants used here are in line with the other odorants mentioned in the protection experiments (Figure 50).
2. Ringer's + 0.5% MeOH, 4 minutes; 10^{-4} M mersalyl, 4 minutes; Ringer's + 0.5% MeOH, 4 minutes. The epithelia was then washed with Ringer's + 0.5% MeOH for 10 minutes. EOGs were elicited by each odorant and n-amyl acetate for the next 40 minutes. The EOG responses to the odorants as well as n-amyl acetate did not recover from the

Fig. 50. Recovery of the ECG responses to butanol, benzyl acetate, nitrobenzene, β -ionone and linalyl acetate from the effects of 10^{-3} M n-amyl acetate in solution. (see text)



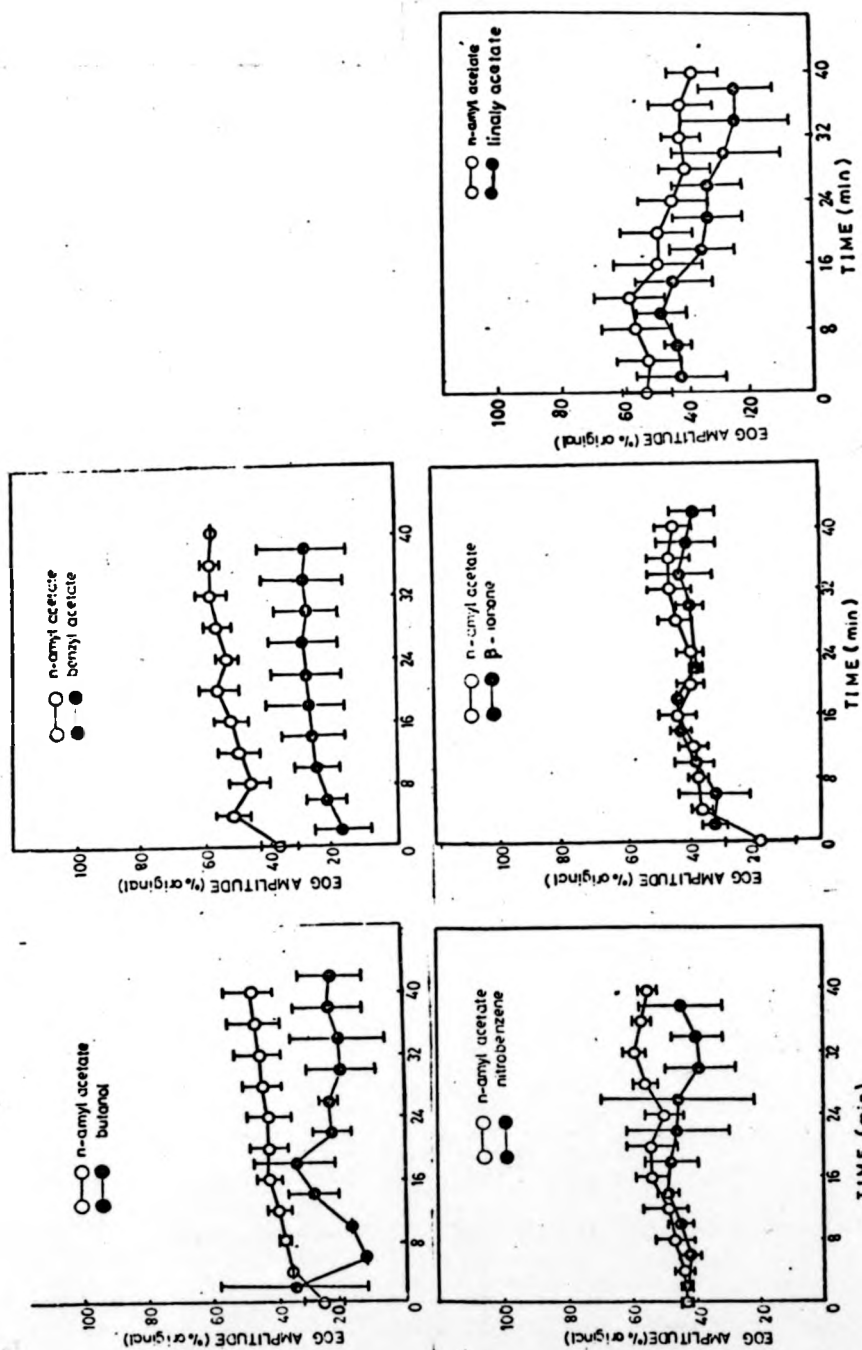


Fig. 51. Effect of 10^{-4} M mersalyl on the EOG responses to butanol, benzyl acetate, nitrobenzene, β -ionone and linalyl acetate.

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Butanol
Benzyl acet
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effect of mersalyl alone (Figure 51). At the end of 38 minutes' recording, the EOG responses recorded upon puffs of the odorants are described in the table below.

| Odour | % Recovery after 38 minutes | No. of frogs |
|-----------------|---------------------------------|--------------|
| Butanol | 23 \pm 11 (mean \pm S.E.M.) | 2 |
| Benzyl acetate | 28 \pm 13 " | 2 |
| Nitrobenzene | 44 \pm 13 " | 2 |
| β -ionone | 41 \pm 9 " | 2 |
| Linalyl acetate | 24 \pm 12 " | 2 |

The treatment of the mucosa with 10^{-4} M mersalyl brought about an irreversible inhibition in the responses to the above odorants as well as to n-amyl acetate.

3. 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH, 4 minutes; 10^{-3} M n-amyl acetate + 10^{-4} M mersalyl in Ringer's + 0.5% MeOH, 4 minutes; 10^{-3} M n-amyl acetate in Ringer's + 0.5% MeOH, 4 minutes. The mucosa was then washed with Ringer's + 0.5% MeOH for 10 minutes. EOGs were elicited by each odorant and n-amyl acetate for the next 40 minutes. The peak amplitudes of these EOGs are represented by the curves for each odorant on each of the graphs. Figure 47 shows that the EOG amplitudes do not recover after treatment with 10^{-4} M mersalyl even if n-amyl acetate is present before, during and after exposure to the protein reagent. Whereas the EOG responses to n-amyl acetate do recover and subsequently the receptors to n-amyl acetate have been

Fig. 51. Effect of 10^{-4} M mersalyl on the EOG responses to butanol, benzyl acetate, nitrobenzene, β -ionone and linalyl acetate.

protected by n-amyl acetate in solution against the effect of
mersalyl, n-amyl acetate in solution did not protect the receptors
to the odorants^{butanol} benzyl acetate, nitrobenzene, β -ionone and linalyl
acetate.

3.8 DISCUSSION - PART B

Odour discrimination by vertebrate olfactory receptors is postulated as the interaction of molecules with the receptor cell membrane. If coupled with specific ion conductance mechanisms, this interaction may result in the generator potential. When the generator potential reaches a certain threshold, action potentials are in turn generated by the receptor cell that transmits sensory information to the nervous system. It is assumed that signals preserve some informational content about the characteristics of the odorant molecule. Therefore, the discriminatory power of the olfactory system cannot be explained satisfactorily without the existence of a number of receptor sites, each of which is specifically capable of physical interactions with a range of stimulant structures.

In all chemoreceptor processes, interaction mechanisms and informational organisations are similar. In all processes, molecular shape, size and molecular orientation in the interaction complex, may be the information-carrying details.

The first direct evidence of proteinaceous receptor sites that odorants exist in the olfactory chemosensitive membrane, and that these sites are specialised for binding with different types of odorants in frogs, was obtained by Getchell and Gesteland (1972).

Further evidence that the receptor sites for odorant molecules

are proteins has been obtained in this thesis by the study of effects of group-specific protein reagents on the characteristics of the EOG. A variety of group-specific reagents, Nbf-Cl, FDNB, TNBS, pCMB, NEM and mersalyl, reduced the EOG amplitude and altered its waveform or abolished it.

Reduction or abolition of the EOG by group-specific reagents could be attributed to three possible actions of these reagents: 1) interaction with olfactory receptor proteins; 2) inhibition of a system which is involved in the ion conductance; 3) non-specific disruption of membrane proteins. The results of the protection experiments cannot be explained by the third type of non-specific effect.

The EOG response is the summation of the generator potentials of all excited chemoreceptors on the olfactory eminence of the frog (Ottoson, 1971). The information content of such a summation potential is poorly understood. However, the EOG has been considered to be the waveform resulting from two processes occurring simultaneously in the receptor cell membrane. Firstly, a long-lasting negative-going process, mainly reflecting an increase in the permeability of the receptor cell membrane to Na^+ . And secondly, a positive-going process of shorter duration due to an increased permeability to K^+ and Cl^- ions, (these are closer to equilibrium with the cell membrane potential, which results in a smaller current flow than in the first case (Gesteland, 1964). The influence of different extracellular ionic media has also been investigated (Takagi *et al.*, 1968). It has been reported that EOG depolarisation (excitation) depended on the

presence of Na^+ ions and that a similar correlation existed between K^+ , Cl^- and hyperpolarisation (inhibition). The resulting slow potential thus reflects the interplay between excitatory and inhibitory mechanisms. Therefore, it can be assumed that ion conductances for the olfactory transduction ~~are~~ carried out by a system which causes ion fluxes across the membrane upon odour stimulation. If the receptor and the system involved in the ion fluxes were one and the same protein, it would not ^{be} possible to explain why interactions resulting in excitatory processes were protected by the odorant in solution while interactions resulting in inhibitory processes were not. Selective and irreversible reductions on the EOGs to Nbf-Cl, but not the EOGs to other odorants also rules out the possibility that the system and receptor are the same molecule (Figure 37). It can be considered that the neural membrane under the control of odorant molecules comprises two elements of structure: the receptor proteins which carry receptor sites with different shape, size and polarity for a different class of odorant molecules, and a system involved in the generation of the generator potentials. As will be indicated in the appendix , the system may include an adenylate cyclase. Its involvement in hormone-receptor interactions has been established (Robison et al., 1971). The odour receptor and the system would play a role similar to that of the regulatory and catalytic subunit in this enzyme, i.e. adenylate cyclase. Coupling between the receptor and the system can be viewed as an indirect, and thus allosteric interaction mediated by structural transition (Monod et al., 1965). An odorant molecule triggers the change of the membrane property by

its reversible binding to a receptor site where it acts as a regulatory ligand. Therefore a reduction or abolition of the EOGs by group-specific protein reagents can only be attributed to the interaction with olfactory receptor proteins.

The result obtained in the protection experiments can be explained as follows:

All the receptor sites for n-amyl acetate were protected by the odorant from the effects of mersalyl. The linkage between the n-amyl acetate receptors and the system which governs ion permeability was not affected either. That is, the message is received from the receptor (n-amyl acetate-discriminator) through a transducer to the system (an amplifier); thus n-amyl acetate receptors function normally. It appeared that n-amyl acetate protects receptor sites, for some odorants, but not others, from the effects of mersalyl. These results imply that n-amyl acetate may interact with more than one type of receptor site. The lack of protection of odorants by n-amyl acetate in solution against mersalyl results in the inhibition of their receptor sites.

1. GROUP-SPECIFIC PROTEIN REAGENTS

The study of the group-specific protein reagents on the characteristics of the EOG has indicated that specific effects depended on the reagents, their concentration and duration of exposure to the epithelium and the stimulus used. The consequences of modification of sulfhydryl groups have been studied with several reagents; DTNB, NEM, mersalyl, FDNB and pCMB. DTNB, which reacts with sulfhydryl groups with high specificity, had no effect on the EOGs. DTNB is a rather large compound, and bears a net charge of -2. Its lack of activity could be due to its inaccessibility to a critical membrane site, because of size, electrostatic effects, or lack of permeability through the mucus layer. Conversely NEM is smaller and neutral, and is known to be a penetrant reagent (Jacob and Janal, 1962; Vignais and Vignais, 1973).

NEM irreversibly blocked the EOG response to n-amyl acetate (Figure 40). It has been shown that this effect of NEM could be prevented by the presence of ethyl n-butyrate in solution (Getchell and Gesteland). They interpreted this results as the specific interaction of NEM with sulfhydryl groups of the receptor proteins located at the exterior surface of the membrane. With respect to its permeability through the cell membrane, one could argue that the effects of NEM may not be specifically directed at the receptors at the exterior surface of the cell membrane. It may well act as a non-specific reagent on the system(s) involved in the ion conductance after passing

through the cell membrane.

The application of TNBS at a high concentration had little effect on the EOGs to n-amyl acetate and 1,8 cineole. TNBS, which selectively reacts with amino and sulfhydryl groups in proteins, reduced the EOGs to odorants by about 20% of their original values. The application of TNBS at the same concentration (10 mM) to an olfactory mucosa which had been treated with a proteolytic enzyme, trypsin, irreversibly abolished the EOGs to the odorants n-amyl acetate and 1,8 cineole. This may indicate that the receptor sites involved in the binding processes with odorants may become susceptible to the action of TNBS after proteolytic enzyme digestion.

The effects of DTT in these experiments depended on the concentration of the reagent and the amount of the mucus present on the olfactory eminence of the frog. However, the use of high concentration (10 mM) of the reagent reduced the EOGs by about 30% of the original amplitude. This small inhibition brought about by the sulfhydryl reducing reagent, DTT, was reversed by the subsequent application of an oxidising agent, DTNB.

Similar experiments with sulfhydryl reagents were carried out using the motor end-plate region of chick muscle (Rang and Ritter, 1971). It was reported that the reduction of the receptor by DTT made the tissue less sensitive to carbachol and acetylcholine. These effects were fully reversed by reoxidation with DTNB.

The application of pCMB as a sulfhydryl group reagent abolished the EOG to n-amyl acetate and decreased the EOG to 1,8 cineole by 95% at the end of a 35 minute exposure of the olfactory epithelium to 1 mM pCMB. This may also indicate the possible involvement of -SH group in the odour receptor interactions (Figure 39).

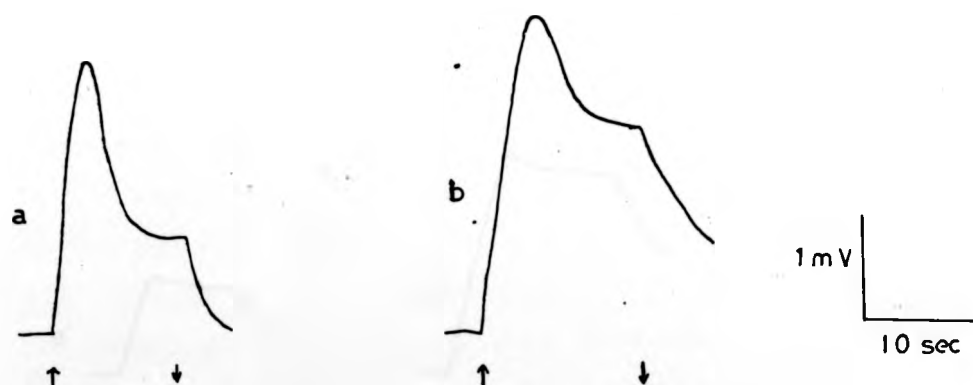
The effects of Nbf-Cl as a group-specific reagent on the EOGs to itself is an interesting phenomenon. Increases in the number of exposures (i.e. number of 10 - 20 second duration puffs) of this reagent to the olfactory mucosa indicated that there is a reduction on the EOG to this reagent, but not to the control odorants. This may indicate that the receptor to this compound is different from those of the control odorants. Although it is reported to be an irritant (Aldrich Chemical Co., Ltd.), the EOG waveform of this reagent with respect to the peak and plateau components plus the rate of rise and decay is similar to the EOGs of the control odorants (Figure 36a). The irreversibility of the responses to this reagent can be attributed (viz the work in which it modified one tyrosine residue per enzyme molecule, the mitochondrial ATPase, (Ferguson *et al.*, 1975)), to the modification of a tyrosine residue in the receptor site of the protein responsible for its specific binding.

FDNB forms covalent bonds with amino-acid side chains containing amino, sulfhydryl, histidyl, and tyrosyl groups, and with membrane lipids (Cooke *et al.*, 1968). The application of FDNB on to the olfactory mucosa in vapour phase resulted in a response in which the

peak and plateau components of the slow potential change are not seen (Figure 38). The rate of rising phase was slow and continued rising to the end of puffing. This abnormal EOG may be caused by effects on the permeability of ions involved in the EOG shape. Puffings of this reagent along with 1,8 cineole resulted in the following: after 100 minutes of experimenting with two successive puffings (with the duration time of 5 minutes and 15 minutes respectively), mean reductions in the responses to n-amyl acetate and 1,8 cineole were found to be different. The response to n-amyl acetate was decreased by about 60% but the reduction for 1,8 cineole was only about 22% of the control response.

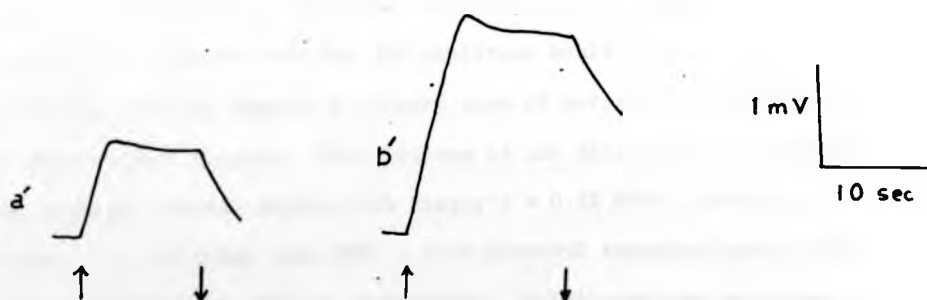
The EOG waveform to n-amyl acetate is somewhat different to that of 1,8 cineole. This is observed in the Figure 52 below, where a) belongs to n-amyl acetate, b) to 1,8 cineole.

Fig.52.



When the EOGs to n-amyl acetate and 1,8 cineole are examined, one always finds that the peak to plateau ratios of the EOGs to these compounds are different. The ratio for n-amyl acetate is between two and three, but for 1,8 cineole this ratio never reaches two. The rate of rise of the slow potential change to both odorants is fast, the rate of decay of the EOG to n-amyl acetate is again fast but for 1,8 cineole this is slow as observed in Figure 52b. Modification experiments with the use of proteolytic enzymes and some group specific reagents resulted in more reductions in the EOG amplitudes to n-amyl acetate than to those of 1,8 cineole. A typical effect of these reagents begins with the reduction of the peak component of the EOG. When the peak falls to the plateau level i.e. the peak to plateau ratio becomes one, the EOG to n-amyl acetate is reduced more than that of 1,8 cineole (Figure 53).

Fig. 53.



Where 'a' shows the typical EOG to n-amyl acetate after the treatment of the mucosa with modification reagents, 'b' represents this situation for 1,8 cineole. It has been noticed that the reduction of the plateau component is difficult and depends on the modifying agent and its concentration. Thus the EOG to n-amyl acetate is more easily reduced than that of 1,8 cineole.

The action of an organic mercurial compound, mersalyl, on the EOG responses to odorants is rapid and the degree of inhibition depends on the concentration of the reagent. The effects of mersalyl were due to its affinity for sulfhydryl groups. Its action is not tissue-specific and is always directed towards the blockage of free, reactive -SH groups of proteins (Mavrier and Hanoune, 1975). They reported that mersalyl, unlike other -SH reagents, was non-penetrant and only acted at the membrane surface. It is therefore certain that mersalyl binds with an odour-receptor situated at the surface of the olfactory neuranol membrane. In Figure 41, the EOG response to n-amyl acetate shows that Ringer's + 0.5% MeOH alone has no effect, but there is a concentration dependent inhibition due to the incubation of the olfactory eminence with mersalyl. The most obvious mechanism for the irreversible reduction of the EOG amplitude would be the formation of a covalent linkage between a mercury atom of mersalyl and the SH group of the receptor protein. This linkage is not dissociable by washing the mersalyl treated mucosa with Ringer's + 0.5% MeOH. However, Figure 43 indicates that DTT, a very powerful reducing agent, was able to reverse the effects of mersalyl, bringing up the responses to

65% of the original amplitude.

This non-penetrant -SH reagent (mersalyl) was shown to react with surface -SH groups involved in the Ca^{2+} -activated ATPase when sarcoplasmic reticulum membranes were incubated in its presence (Hasselbach and Seraydarian, 1966). Similar results were reported in human red blood cells where both the Ca^{2+} -ATPase and the calcium pump were inhibited by mersalyl (Schatzmann and Vincenzi, 1969).

Recently, it was reported that mersalyl inhibited an enzyme, adenylate cyclase, from rat-liver plasma membrane; at 0.1 mM mersalyl completely stopped cyclic AMP (cAMP) formation. The inhibition brought about by mersalyl was thought to be due to both a decrease of the maximal velocity of the reaction and of the affinity of the enzyme for the substrate. It was immediate and irreversible spontaneously, but it was reversed by the simultaneous addition of 2-mercaptoethanol, in a dose-dependent fashion (Mavrier and Hanoune, 1975). Furthermore, mersalyl exhibited maximal inhibition within one minute of being added, independent of concentration, which ranged from 1 to 20 μM . These results are somewhat similar to the effects of mersalyl on the EOG responses found in this thesis. Mersalyl showed the greater part of its inhibition within a minute. Inhibition was completed within 9 - 10 minutes of mersalyl addition and its action, at concentrations of 10^{-2} to 10^{-5} M was not enhanced by increasing the incubation period by up to 20 minutes (Figure 41).

The effects of mersalyl on the EOG amplitudes of all the odorants used in protection experiments were the same. As Figure 42 describes, the peak and plateau components of the EOG are decreased in parallel. This is different to the effect of other modifying reagents which first reduced the peak and then the plateau.

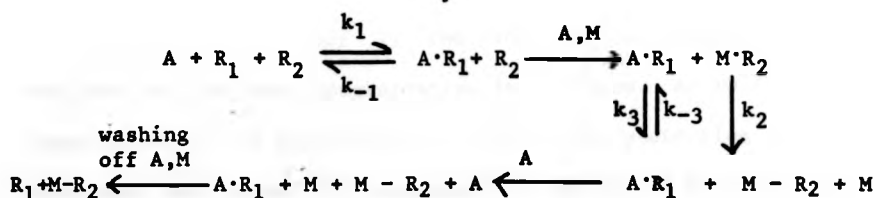
The effects of some of the group-specific reagents used in this work cannot be ascribed to their interaction with any one type of amino acid side chain. Some are relatively non-specific. However, mersalyl specifically reacts with -SH groups of proteins located at the surface of membranes. This indicates that such sulfhydryl groups can be involved in the binding of an odorant with its receptor. Furthermore, the action of DTT (which specifically reduces disulfide linkages to sulfhydryl groups) on mersalyl-treated mucosa, indicated that most of the effects of mersalyl are due to its interaction with sulfhydryl groups which play a major role in the interaction of an odour stimuli with its receptor site in the changes leading to the generation of the EOG.

2- PROTECTION EXPERIMENTS

The group-specific protein reagents react with all accessible membrane proteins. In order to show that the observed effects of these reagents on the EOGs was due to interaction with receptor proteins, a technique analogous to substrate protection of an enzyme active site from an inhibitor has been used.

This technique consists of saturating the receptor proteins with an odorant in solution, applying the protein reagent plus the odorant in solution for a certain period of time, during which the protein reagent will react with all unsaturated proteins, and afterwards unreacted inhibitor is washed away with the odorant in solution.

A kinetic scheme involved in this technique is described below, where A is an odour in solution, R_1 is one kind of receptor, R_2 represents the remainder of the receptors and M is a modifying agent (mersalyl).



An odour A (n-amyl acetate) in solution binds with the receptor, R_1 and forms the complex $A \cdot R_1$. The other receptors represented by R_2 are free to the action of M (mersalyl) and therefore M binds with the

receptors and forms an irreversible complex ($M - R_2$) with the rate constant k_2 . In the last stage, the $A \cdot R_1$ complex prevents the action of excess mersalyl molecules on R_1 . Finally, the excess of A and M are washed away and the free R_1 and $M - R_2$ face to the odour molecules in vapour phase.

The results obtained indicate that n-amyl acetate in solution was able to protect its receptor sites from the irreversible action of mersalyl. Two possible explanations can be made of these protection experiments:

- a) with the use of n-amyl acetate as a protecting agent, the receptor sites for the odorant were isolated (complex $A \cdot R_1$) from the effect of mersalyl (i.e. mersalyl could not affect those sites which were already occupied by n-amyl acetate in solution).
- b) n-Amyl acetate in solution depolarises cells that bear receptor sites for it, the receptor protein undergoes a conformational change, preventing the groups on the protein reacting with mersalyl.

As is shown in Figure 45 the protection experiments were dependent on the odour concentration in solution. At 10^{-2} M and 10^{-3} M n-amyl acetate, the mean recovery value of the protection obtained at 40 minutes was 97% and 89% respectively. With 10^{-4} M n-amyl acetate in solution the final recovery was around 60% of the original value.

The range of specificity of n-amyl acetate receptor sites has been studied. With n-amyl acetate as the protecting substance in

solution and the odorants and n-amyl acetate as the vapour-phase stimulus, the results shown in Figure 46 were obtained. n-Amyl acetate protected the sites for butyric acid, n-butyl acetate, 1,8 cineole phenylacetaldehyde as well as protecting its own sites. Therefore, it may be assumed that these odorants used the same receptor sites. There are no consistent structural and functional group similarities between these odorants and n-amyl acetate. The odour responses elicited by these compounds are also diverse. However, there is a structural and sensory similarity between n-amyl acetate and n-butyl acetate; both are protected floral odorants. But other floral odorants, such as linalyl acetate and benzyl acetate, were not protected by the floral odorant, n-amyl acetate, when they were applied in vapour phase.

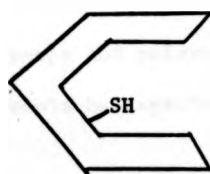
When odorants with different smells and structures were used as the vapour-phase stimuli and n-amyl acetate as the protecting odorant in solution, the EOGs to these odorants showed recovery as well as n-amyl acetate (Figure 46). But the EOGs to butanol, nitrobenzene and β -ionone did not recover to any extent above that attained when n-amyl acetate alone was applied to the mucosa (Figure 47). This indicates that n-amyl acetate and these odorants interact with different receptor sites.

The results imply that n-amyl acetate may interact with more than one type of receptor site. Theories based on the interaction of odorants with more than one type of receptor site have been discussed (Beets, 1973).

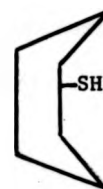
The results also suggest that compounds with dissimilar odours might interact with common receptor sites.

The finding that an odorant can interact with more than one type of receptor site may explain why odour primaries have been difficult to define. Those primaries tentatively identified by Amoore, (1970) may be odorants that interact with only a very few types of receptor site, with one type predominating. Smaller odorants, such as n-amyl acetate, present a less well-defined shape that might interact with many available receptor sites; presumably the functional group(s) in such a molecule would play a large role in determining the types of sites.

As was pointed out earlier, n-amyl acetate might have a high number of receptor sites specialised by its low affinity and less discriminating binding specificity. This is shown below.



High affinity



Low affinity

Receptor sites for odorants, therefore, may be divided into common and specialised sites depending on their affinity. A common receptor site would be of a low affinity and discrimination, and alignment for odorants would be expected to be less critical; the EOG responses to

these odorants would be high in terms of millivolt amplitude. n-Amyl acetate which protects its receptors from the effect of mersalyl, elicits high EOGs. The odorants which had protection of n-amyl acetate in solution against the action of mersalyl also elicit high EOGs. This would indicate that these odorants may use sites as defined common receptor sites which are of a low affinity. On the other hand, odorants which elicit small EOGs may use high discrimination, high affinity sites. Such a situation might exist with certain musks: relatively large molecules that probably require a complex arrangement of receptor protein surface. The recorded responses to musks indicated that they elicit relatively small EOGs.

Recent studies have attempted to relate odour quality to one or two physico-chemical parameters (G. H. Dodd, 1976). It is likely that, if each odorant interacts with a variety of receptor site types, a variety of intermolecular forces and aspects of molecular size and shape would be important in the interactions. An odour-induced effect, that is the EOG, can therefore be a function of the size, shape and polarity of a molecule. Thus no one physico-chemical parameter would be expected as the determinant of odour quality.

3.9 PHOTOAFFINITY LABELLING STUDIES

1- EXPERIMENTAL

- a) General - All melting temperatures were determined with an Electrothermal melting point apparatus and are uncorrected. Proton magnetic resonance spectra were determined on a Perkin-Elmer R12 instrument. Chemical shift data are given as δ units using tetramethylsilane as an internal standard. Infra-red spectra were recorded on a Perkin-Elmer 457 grating spectrophotometer. Ultraviolet spectra were recorded on a Unicam SP1300 spectrophotometer, using the appropriate blank. Microanalyses were performed by the Butterworth Microanalytical Consultancy Ltd., Middlesex, for the aryl azides; p-azido phenyl-acetic acid and p-azido phenyl ethanol. Mass spectra were determined with a Microsmass 12 of V.G. Micromass Ltd.

Exposure to daylight causes rather rapid decomposition of the azides. Syntheses were therefore carried out in a dark area with a dim-red light only. TLC was done on silica gel plates, Merck F254. TLC spots were located under uv light. In addition ninhydrin reagent was used. Purification of the azides were carried out using column chromatography on silica gel (100 - 200 mesh).

- b) Light Source - Photoaffinity experiments were carried out with a HPK 125 W mercury lamp (Philips) as a light source mounted in a Bausch and Lomb housing. To this a filter (18A, Philips, selecting 310 - 350 nm region) was attached. The lamp was 50 cm away from the olfactory mucosa of the frog.

- c) Control experiments - For experiments with BSA, 2 mg/ml of BSA in 0.1 M tris buffer was mixed with 1-azidonaphthalene (3×10^{-5} M in EtOH) in the dark. The excitation spectrum (290 nm) was determined for the protein. The emission spectrum was then recorded. Both spectra were carried out with Perkin-Elmer fluorescence Spectrophotometer MPF-3. The protein plus the azide in solution was irradiated in a quartz cuvette of 1 cm path length, centred in the light beam of 325 nm in the Perkin-Elmer MPF-3 fluorescence spectrometer with its excitation slit fully open (40 nm). At zero, 1 minute, 3 minute, 5 minute, 10 minute and 15 minute irradiations, aliquots were taken from the cuvette and passed through a G25 Sephadex column.

For the experiments in which the solution of azides was irradiated, the light source was again the same as in the above experiments.

- d) Chemicals - α -Naphthylamine was obtained from the Sigma Chemical Co., Ltd. 1-Amino-4 nitronaphthalene and p -amino phenylacetic acid were purchased from the Aldrich Chemical Co., Ltd. Other chemicals used as precursors of the azides and involved in the synthesis of the azides were obtained from the Koch Light, Aldrich and Sigma Co., Ltd. and were analytical reagent grade.

- e) Synthesis of photoaffinity labels

The azides synthesised from the corresponding amines by method I of Smith and Brown (1951) were; 1-azidonaphthalene, 1-azido 4-nitronaphthalene, p -azidophenylacetic acid, p -azido phenylethanol and p -azido nitrobenzene. The method for the synthesis of these azides

consisted of diazotisation of a precursor amine followed by treatment with sodium azide.

Phenylazide was synthesised according to the method described by Fieser and Fieser (1967). Phenyl hydrazine was the precursor for phenylazide.

Compounds 1-azidonaphthalene, 1-azido 4-nitronaphthalene, p-azido nitrobenzene and phenylazide were previously known and their spectroscopic characteristics were similar to those in the literature.

p-Azido phenyl acetic acid and p-azido phenylethanol, which were not previously known, were identified on the basis of their micro analysis and their spectroscopic characteristics.

- 1) p-Azido phenylacetic acid - was synthesised by diazotisation of the corresponding amine (p-amino phenylacetic acid) $[HCl, NaNO_2]$ followed by addition of NaN_3 . p-Amino phenylacetic acid yielded a light yellow solid (5.9 g, 78%); m.p. $84^\circ C$; NMR ($CHCl_3$), 3.49 (CH_2) 7.20 (aromatic), 10.2 (OH); infra-red spectrum (film), 2120 ($-N_3$); ultraviolet spectrum (Hexane) λ_{max} 254, 282, and 291 nm.

$C_8 H_7 N_3 O_2$

Calculated: C, 54.23; H, 3.98; N, 23.72

Found: C, 54.52; H, 4.16; N, 23.48

- 2) p-Azido phenylethanol - was synthesised by diazotisation of the corresponding amine p-aminophenyl ethanol ($HCl, NaNO_2$) followed by addition of $NaNO_3$. p-Aminophenyl ethanol yielded a yellow oil, NMR ($CDCl_3$), 2.83 (ϕCH_2), 3.83 (CH_2OH), and 7.20 S (aromatic); IR (film) 2125 ($-N_3$) and 1050 cm^{-1} ($-CH_2OH$); UV (methanol), λ_{max} 250, 280 and 290 nm.

$C_8 H_9 N_3 O$

Calculated: C, 58.88; H, 5.56; N, 25.75

Found: C, 58.58; H, 5.80; N, 25.46

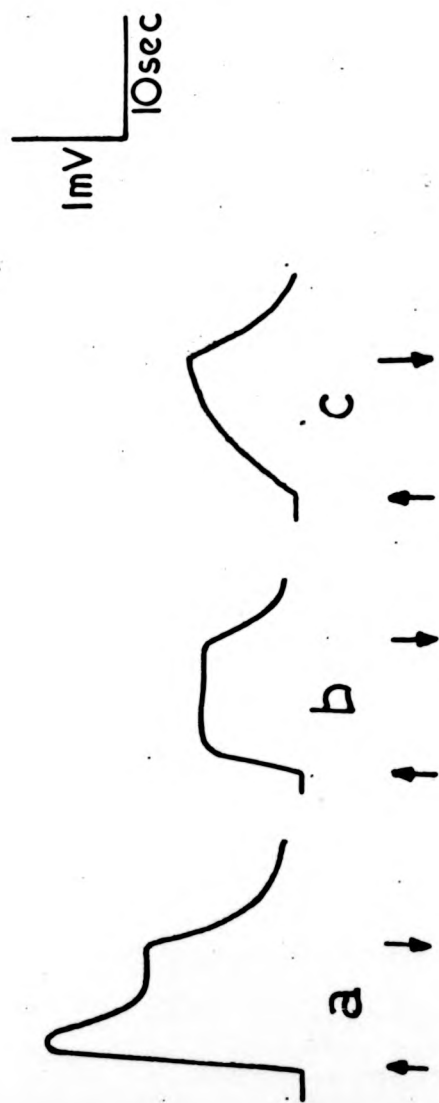


Fig. 54. EOG types observed upon puffs of azides in the dark.

3.10 RESULTS - PART C

Photoaffinity Labelling Studies

In this part of the work, a photoaffinity labelling technique was used in studying the receptors involved in the discrimination of odorants. In this procedure an aryl azide as odorant was allowed to bind to the receptor site. It was then activated photochemically to produce a chemically reactive, short-lived intermediate nitrene that was expected to combine immediately and covalently with any neighbouring structure at the binding region, thus bringing about a permanent inhibition of the receptor. For this purpose aryl azides, described in the methods of this chapter, were synthesised.

I was able to use only three azides, because the rest did not elicit any responses from the frog's olfactory eminence. The azides used in this work were: 1-azidonaphthalene, 1-azido 4-nitronaphthalene and phenylazide.

A- IN THE DARK

Three different EOG types (in terms of waveform) were obtained following puffs of the azides used in this work. These EOG types, shown in Figure 54, are as follows:-

EOG type I - In terms of a peak and plateau appearance this EOG type is similar to the EOGs of control odorants (Figure 54a). The rate of the EOG rise to the peak value is fast. The rate of decline is slower than that of n-amyl acetate, but similar to that of 1,8 cineole. The peak to plateau ratio is always higher than one but, except in a few

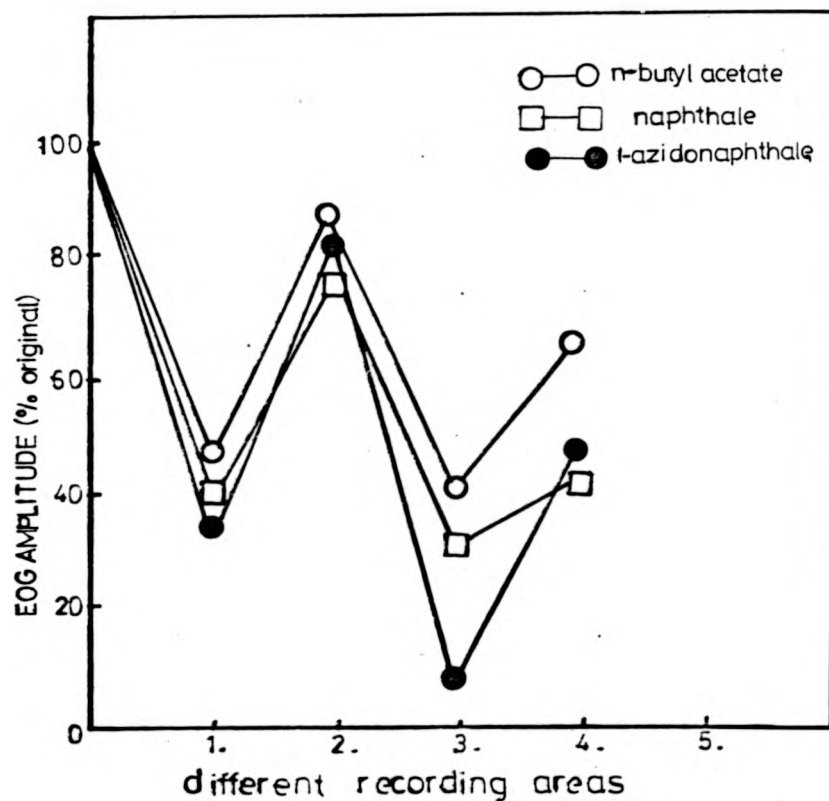


Fig. 55. EOG responses elicited by odorants from different parts of the olfactory mucosa in the dark.

- n-butyl acetate
- naphthale
- t-azidonaphthale

cases, this ratio never reached two (the peak to plateau ratio in the EOGs to n-amyl acetate is always around or higher than two). The EOG type I is elicited by the azides used in this work.

EOG type II - In this type of EOG there is no peak (Figure 54b).

Therefore, the peak to plateau ratio is one. The rate of rise and decay is slower than for type I.

EOG type III - The peak to plateau ratio in this type of EOG is lower than one (Figure 54c). This has been calculated taking the height at 5 seconds as the peak and at 10 seconds as the plateau from the beginning of stimulation. That is, when a 10-second puff is given, the peak is the one which appears in the first five seconds and the plateau is in the last 5 seconds. The rate of rise and decline is slower than the two previous EOG types. This type of EOG is rarely observed.

The waveform of EOGs elicited by the azides were found to vary, not only from one frog to the other, but also from one location on the frog's olfactory eminence to another.

Incidentally, the EOG amplitude to the control odorants and to the azides showed a similar pattern of response-changes (Figure 55) with electrode position changes over the olfactory mucosa. This may indicate that either they use the same receptors or they have different receptors which are distributed in the same way. Although this particular experiment was conducted in the dark because of the azide, the EOGs elicited by the control odorants followed the same pattern of changes in their amplitude with electrode position changes in the light as well.

butyl acetate
naphthale
azidonaphthale

cases, this ratio never reached two (the peak to plateau ratio in the EOGs to n-amyl acetate is always around or higher than two). The EOG type I is elicited by the azides used in this work.

EOG type II - In this type of EOG there is no peak (Figure 54b).

Therefore, the peak to plateau ratio is one. The rate of rise and decay is slower than for type I.

EOG type III - The peak to plateau ratio in this type of EOG is lower than one (Figure 54c). This has been calculated taking the height at 5 seconds as the peak and at 10 seconds as the plateau from the beginning of stimulation. That is, when a 10-second puff is given, the peak is the one which appears in the first five seconds and the plateau is in the last 5 seconds. The rate of rise and decline is slower than the two previous EOG types. This type of EOG is rarely observed.

The waveform of EOGs elicited by the azides were found to vary, not only from one frog to the other, but also from one location on the frog's olfactory eminence to another.

Incidentally, the EOG amplitude to the control odorants and to the azides showed a similar pattern of response-changes (Figure 55) with electrode position changes over the olfactory mucosa. This may indicate that either they use the same receptors or they have different receptors which are distributed in the same way. Although this particular experiment was conducted in the dark because of the azide, the EOGs elicited by the control odorants followed the same pattern of changes in their amplitude with electrode position changes in the light as well.

5.

5

from different parts

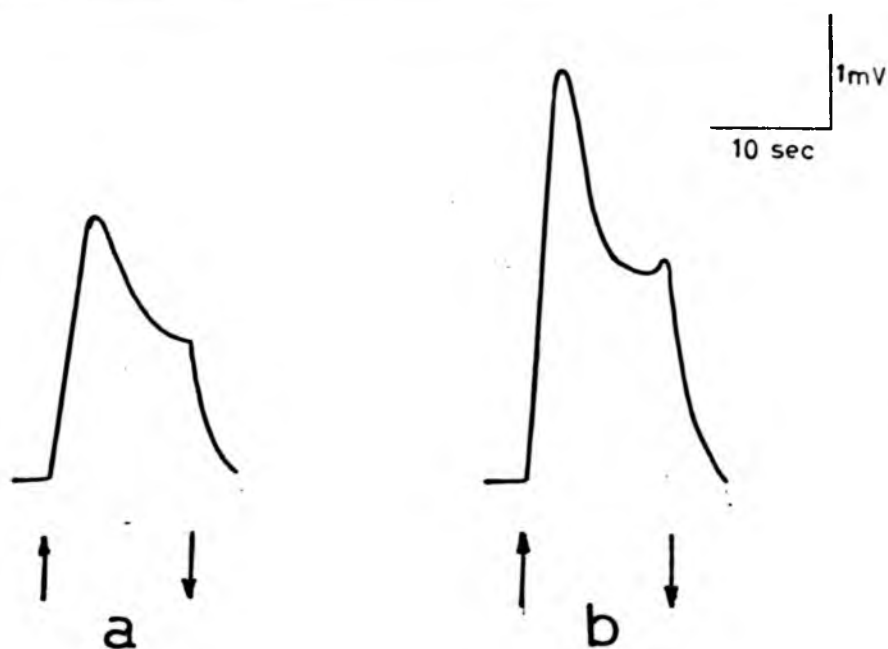


Fig. 56. EOG responses elicited by 1-azidonaphthalene (a) and ethyl n-butyrate (b).

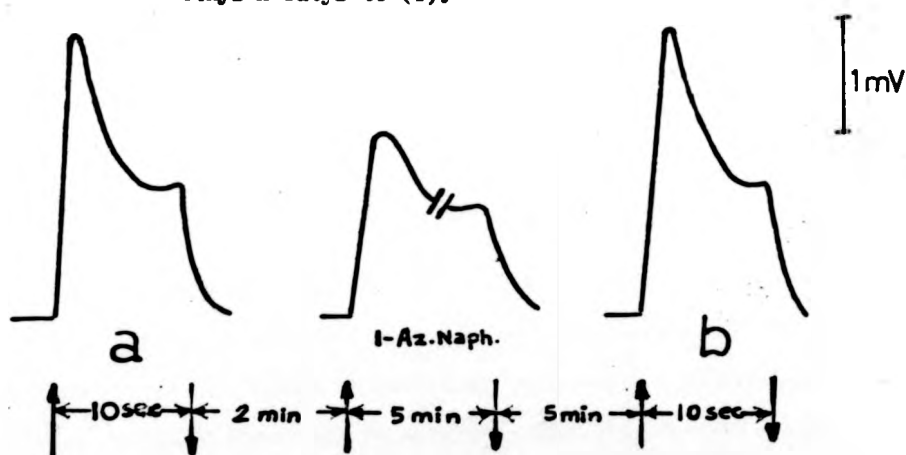


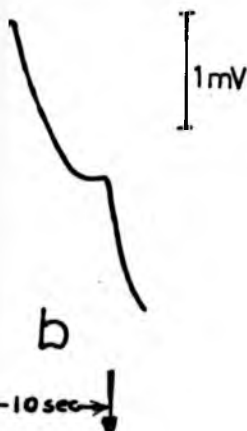
Fig. 57. Effect of 1-azido naphthalene on the EOG responses elicited by n-amyl acetate.

1mV

10 sec



halene (a) and



responses

A typical EOG of 1-azidonaphthalene is shown in Figure 56 (a). It is quite similar to that of ethyl n-butyrate (b).

In the dark, with a good resting time (usually 2 minutes, depending on the pulse length), subsequent puffs of azides evoked similar EOGs in terms of amplitude value and waveform. This was not changed by increasing the pulse length for up to 10 minutes, providing a sufficient rest interval is allowed (i.e. a 15 minute interval between 10 minute pulses). (However, in just one case a dark reaction was observed upon application of 1-azidonaphthalene. EOG responses to this azide were reversibly diminished after receiving several short puffs of 2 - 3 minutes' duration.)

In all cases the amplitudes and waveforms of the control odorant EOGs were unaffected by the azides puffed in the dark. An example of this is shown in Figure 57, where n-amyl acetate elicited a response which is a, 1-azidonaphthalene was then puffed for 5 minutes, followed by a five-minute interval after which n-amyl acetate was again puffed which resulted in response b. It was found that a and b were equal.

These controls showed that there was no side effect of the azides on the mucosa in the dark. Also, puffs of the control odorants with the long duration in the dark made sure that there was no change in the amplitude and waveform of the EOGs.

Finally, all dark experiments were carried out in complete darkness with only a dim-red light as a light source.

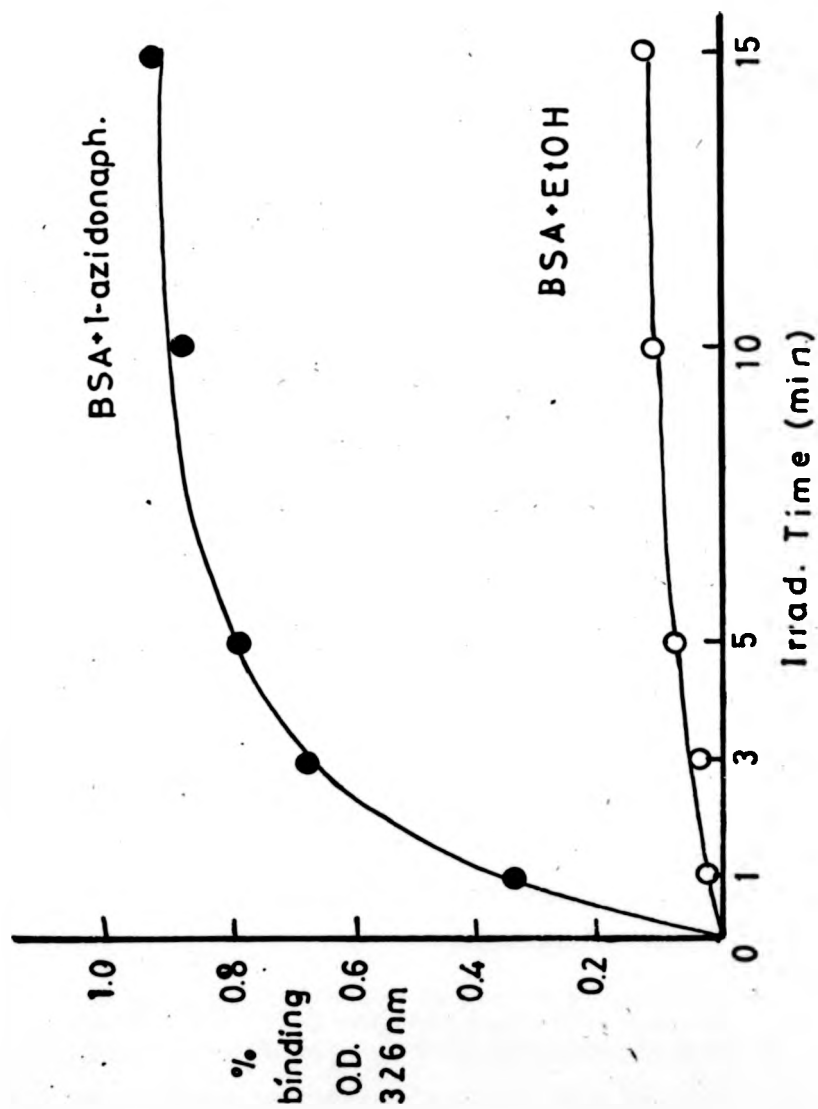


Fig. 59. Binding of 1-azido naphthalene (3×10^{-5} M in EtOH) to BSA (2 mg/ml.).

Fig. 58b . UV absorption spectra of 1-azido 4-nitronaphthalene
(in Hexane) after various times of photolysis. (in mins)

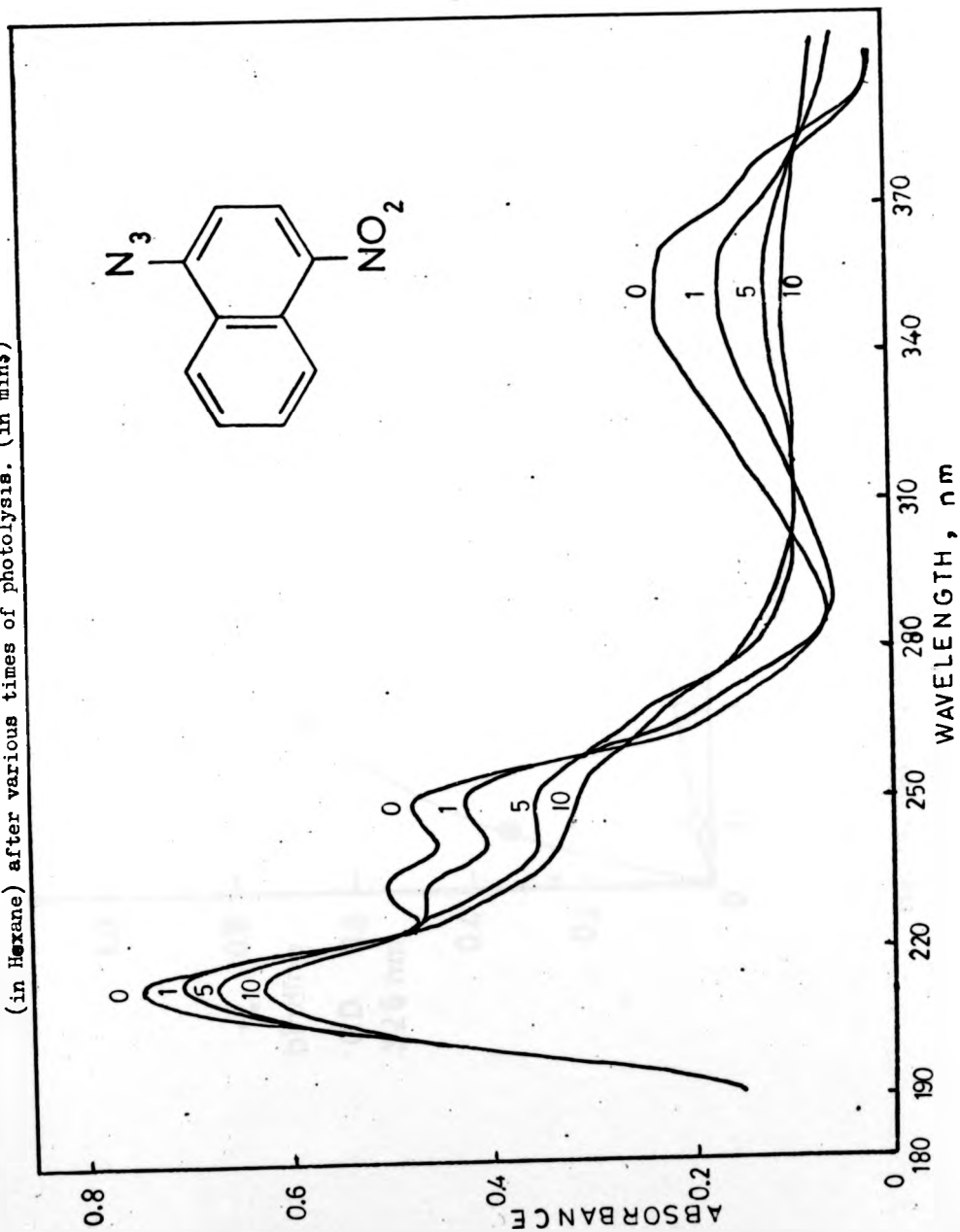
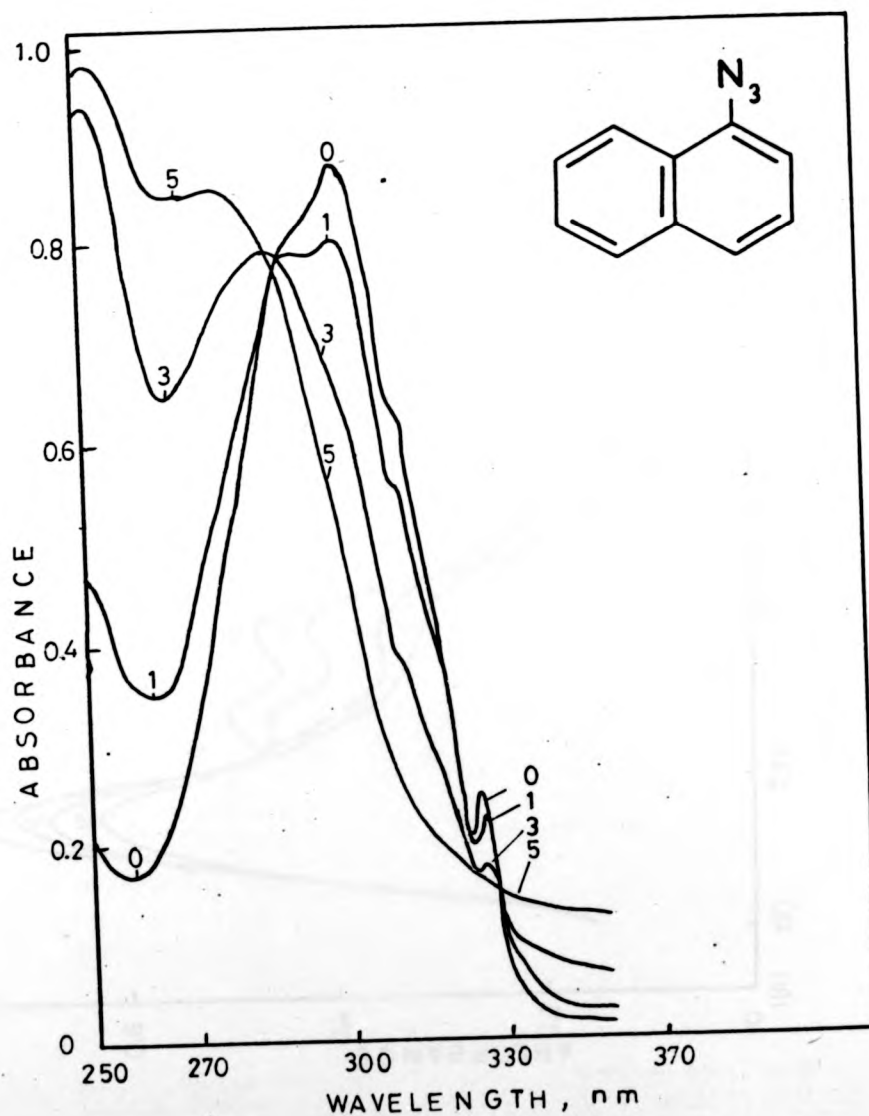


Fig. 58a. UV absorption spectra of 1-azido naphthalene (in Hexane)
after various times of photolysis. (in mins)



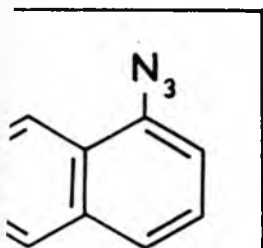
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phthalene (in Hexane)
in mins)



In this way, one of three criteria, chemical inertness of arylazides in the dark, has been satisfied. The others are as follows:- a suitable absorption maximum and an insertion of the photoproduct, nitrene, into a protein.

uv-Absorption spectra of the azides: 1-azidonaphthalene and 1-azido-4-nitronaphthalene, are shown in Figure 58 a, b, respectively. Irradiation of 1-azidonaphthalene in hexane solution at 326 nm (which is due to the $-N_3$ group) changed the spectra of the compound, Figure 58a, indicating that this was a suitable wavelength for decomposing the azide and generation of the nitrene. For 1-azido-4-nitronaphthalene this was found to be 310 nm and above (Figure 58b).

The last criterion has also been checked with the insertion of an azide into a protein. 1-Azidonaphthalene was used as a precursor of the nitrene species and bovine serum albumin (BSA) was the protein. Figure 59 shows the time course of the insertion of this azide into BSA. 2 mg/ml of BSA in 0.1 M tris buffer were irradiated in the presence of 3×10^{-5} M 1-azidonaphthalene. Aliquots were removed at different time intervals and passed in the dark through a Sephadex Q-25 column equilibrated with 0.1 M tris buffer. The data refer to the exclusion peak.

B- IN THE LIGHT

Because the photoaffinity labelling technique requires a light source for converting azides to nitrenes, great attention was paid to the control experiments. These controls are as follows:-

370

nm

1. the effect of light on the olfactory mucosa of the frogs,
2. the effect of light on the responses elicited by control odorants so as to compare responses before and after irradiation,
3. the effect of light while control odour is puffed (the uv is on while the control odour is puffed.)

The light sources used in two different labs had the same power (125 W). A filter was necessary to obtain a certain wavelength for the irradiation of azides. Therefore, that particular wavelength was kept constant for all experiments, the wavelength being above 310 nm.

1. - The effect of light on the olfactory mucosa of frogs

Observations have been made to examine colour and mucosal depth changes. Without the filter, it was observed that the yellowish colour of the olfactory eminence changed to brown within a minute of irradiation. This effect was followed by a decrease in the amount of the mucus covering the olfactory epithelium. With the filter up to ten minutes of irradiation of the eminence did not cause any change in the colour and the amount of mucus. Observations were made through a light microscope.

2. - The effect of light on the responses elicited by control odorants

It was observed that without the filter the EOGs to control odorants, n-amyl acetate and ethyl n-butyrate, were decreased by about 70%. This was the result of 3 - 5 minutes irradiation of the olfactory eminence. There was no recovery even though a 20 - 30 minute interval time was allowed. With the filter, there was no decrease in the EOG

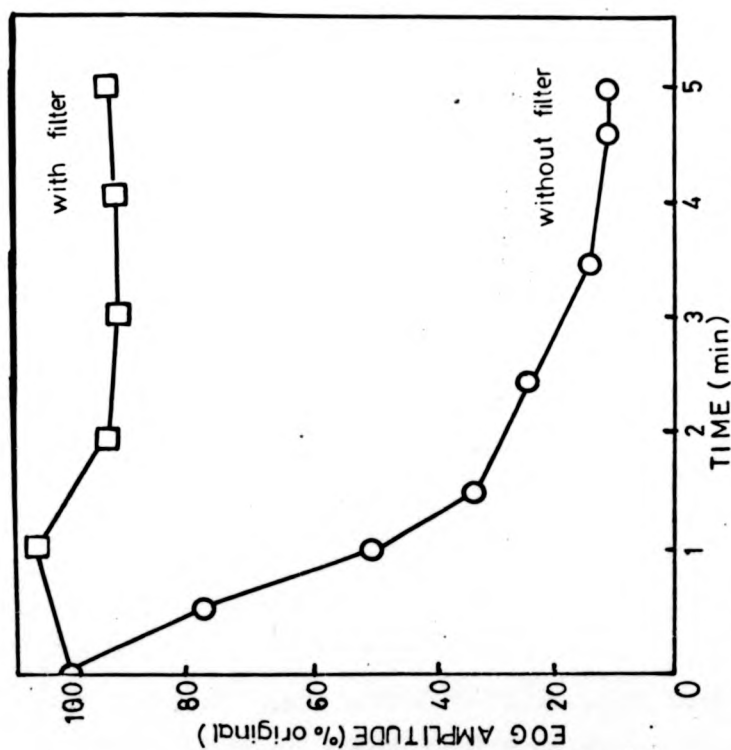


Fig.60 The effect of light on the EOG responses to n-amyl acetate

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amplitude to the control odorants. A typical experiment of this kind is shown in Figure 60. The filter was deemed satisfactory for use in the photoaffinity labelling experiments.

3. - The effect of light applied during the control odorant puff

This control was required because irradiation was to be carried out while the azide was eliciting an EOG response. It was thought that such an irradiation might affect control odour receptors that were in an active state interacting with odorant molecules. In this type of control experiments the irradiation was started 10 seconds after stimulation with observation of the plateau. The duration time for irradiation was up to 10 minutes. The results indicated that there was no effect on the EOG amplitude and waveform.

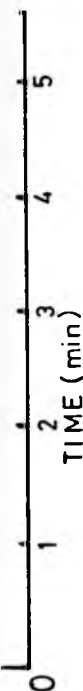
Finally, it was observed that with every irradiation, the light itself changed the resting potential in both directions. The light effect was reversed when the light was switched off.

The effect of light on the responses evoked by azides

Results indicate that the EOGs to the azides were affected by the irradiation. Two effects, namely changes in the EOG amplitude and waveform, were noted.

1. - EOG amplitude to azides was reduced upon photolysis. However, this change was dependent on the EOG type concerned. As has been described previously, there are three types of EOG elicited by azides

Fig.60 The effect of light on the EOG responses to n-amyl acetate



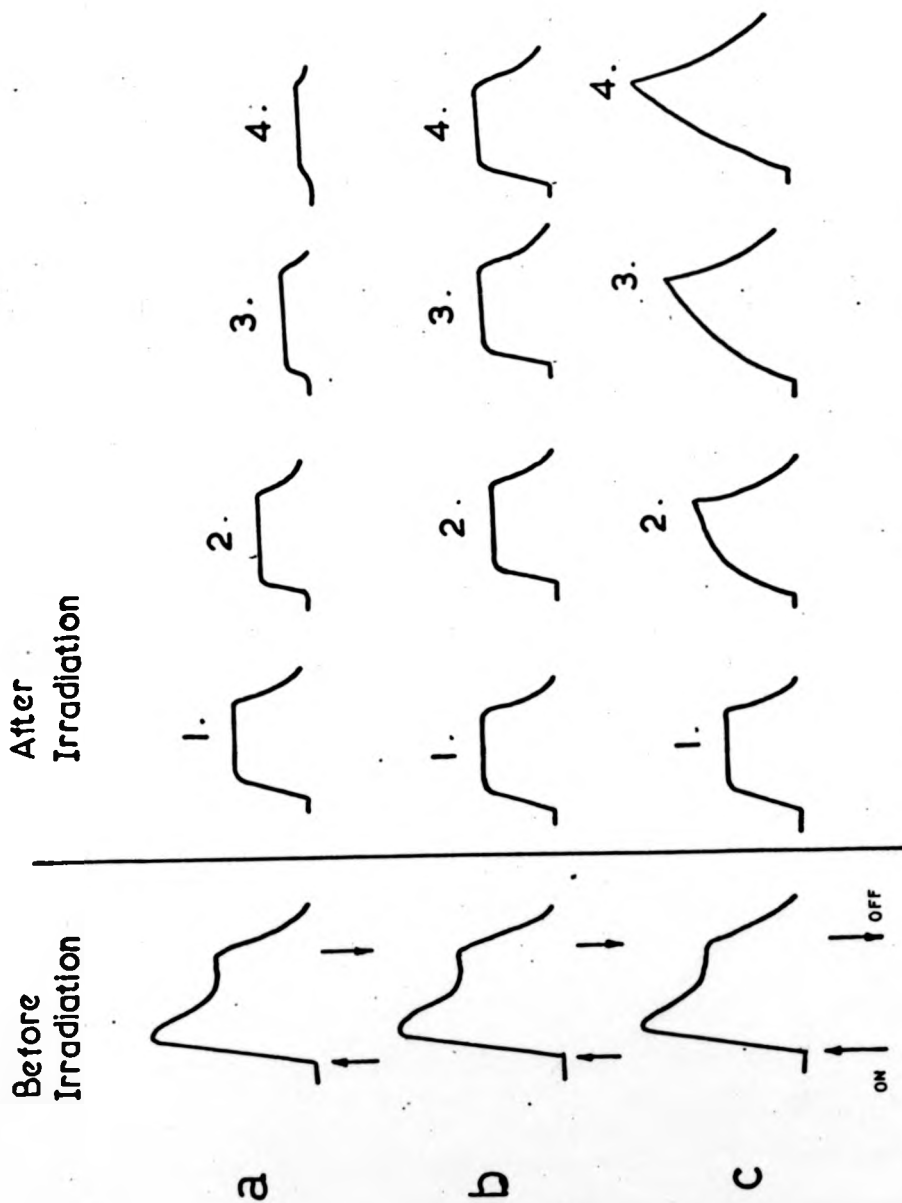


Fig. 61. The effect of light on the EOG responses elicited by azides.

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in the dark (Figure 54). Type I, which had a peak, lost it upon photolysis. With further irradiation reduction of the plateau level was also observed. Three types of light effects on the EOGs to the azides are as follows:-

- a) 100% reduction, and therefore, a zero recorded potential has been made upon an azide puff (Figure 61a and see the results below).
- b) The plateau decreased to a level at which it stayed throughout the experiments (Figure 61b).
- c) After the disappearance of the peak and some parts of the plateau, the EOG type III began to be observed (Figure 61c).

However, the reduction of the EOGs to azides is not consistent and, therefore, reversibility was sometimes observed.

2. - The EOG waveform to azides was changed upon photolysis. As is seen in Figure 61, the rates of rise and decay of the EOGs to the azides became slower.

Apart from the above effects one case showed that the EOG amplitude to 1-azidonaphthalene dropped below the resting potential and displayed a positive potential change after a short time of irradiation. But the result was not irreversible (consider further results).

Because a novel biochemical approach to the olfactory coding mechanism was applied in this work, a presentation of the results would be important. However, as was described previously, the results were equivocal, and they were not consistent in every experiment. Therefore, in the next stage, the graphs presented are the results of each experiment.

Fig. 61. The effect of light on the EOG responses elicited by azides.



a) The effect of light on the responses elicited by 1-azidonaphthalene

1-Azidonaphthalene has been chosen as an aryl azide to label its receptor site specifically for the following reasons:-

a) It has been selected because of its behaviour as a normal stimulant for the frog in the dark.

b) It is a pleasant smelling liquid and can be likened to naphthalene. This would mean that naphthalene could be used as an odorant with a similar smell and structure competing for the same receptor along with this azide.

c) As has previously been described only a short period (1 - 2 minutes) of irradiation was required for decomposition. In this way, the side effects of a longer period of irradiation have been eradicated.

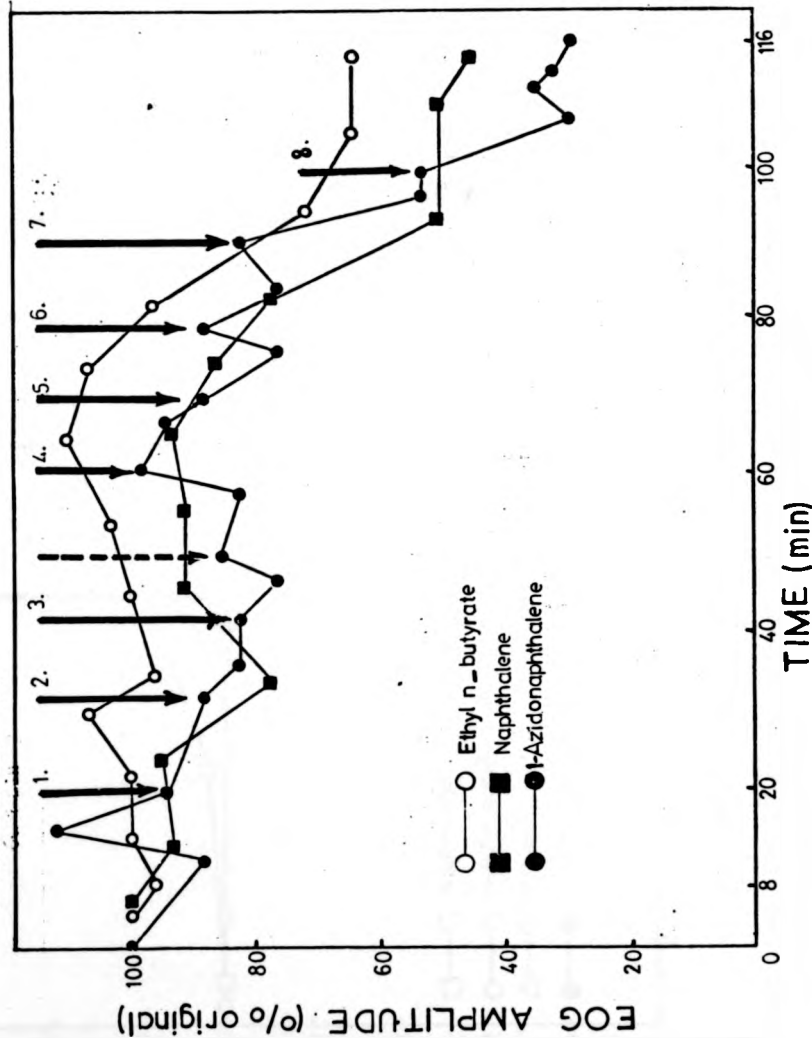
Graph No. 1

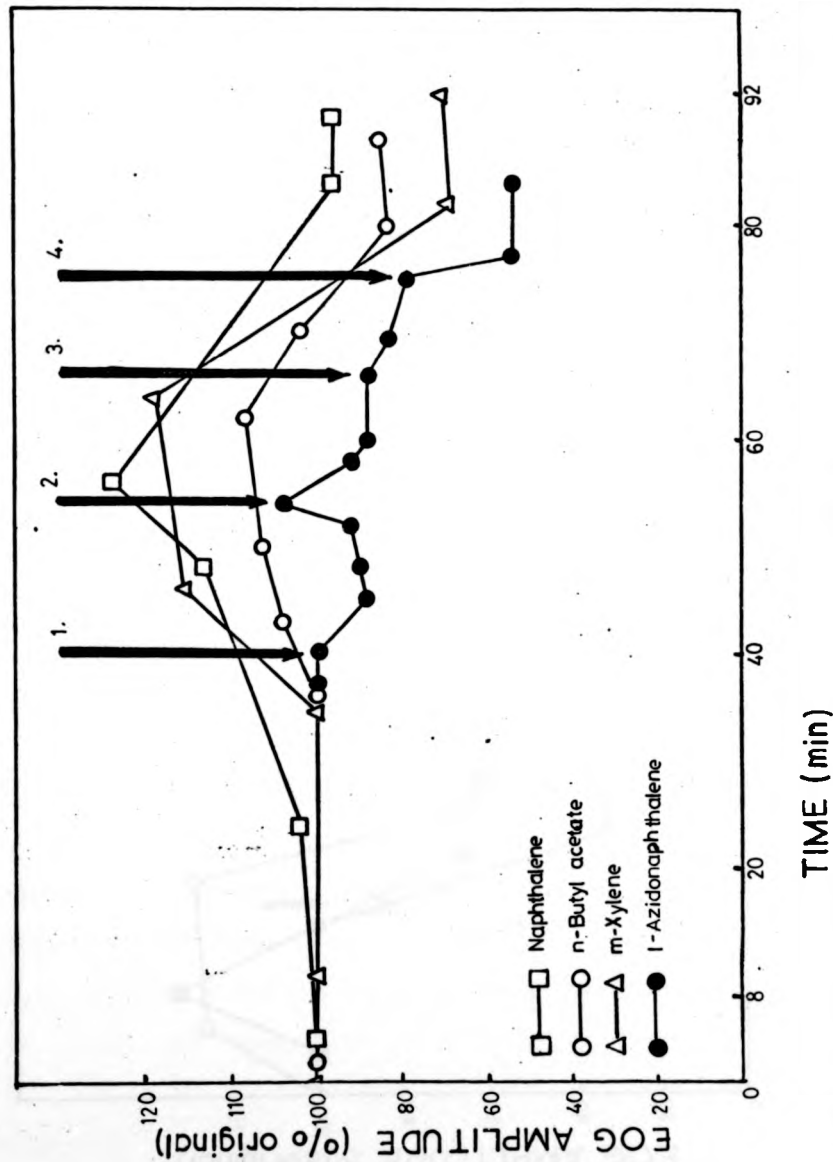
In this work, three compounds, ethyl n-butyrate, naphthalene and 1-azidonaphthalene were applied to the olfactory mucosa in vapour phase with a 10 seconds pulse length and an interval of 2 minutes duration. Reversible reductions of the EOGs to 1-azidonaphthalene were observed with 6 irradiations. With the 7th and 8th irradiations, irreversible reductions were observed in the EOG amplitude to the azide, bringing about a 70% decrease, whereas decreases in the EOGs to the control odorants are 55% for naphthalene and 35% for ethyl n-butyrate.

Graph No. 2

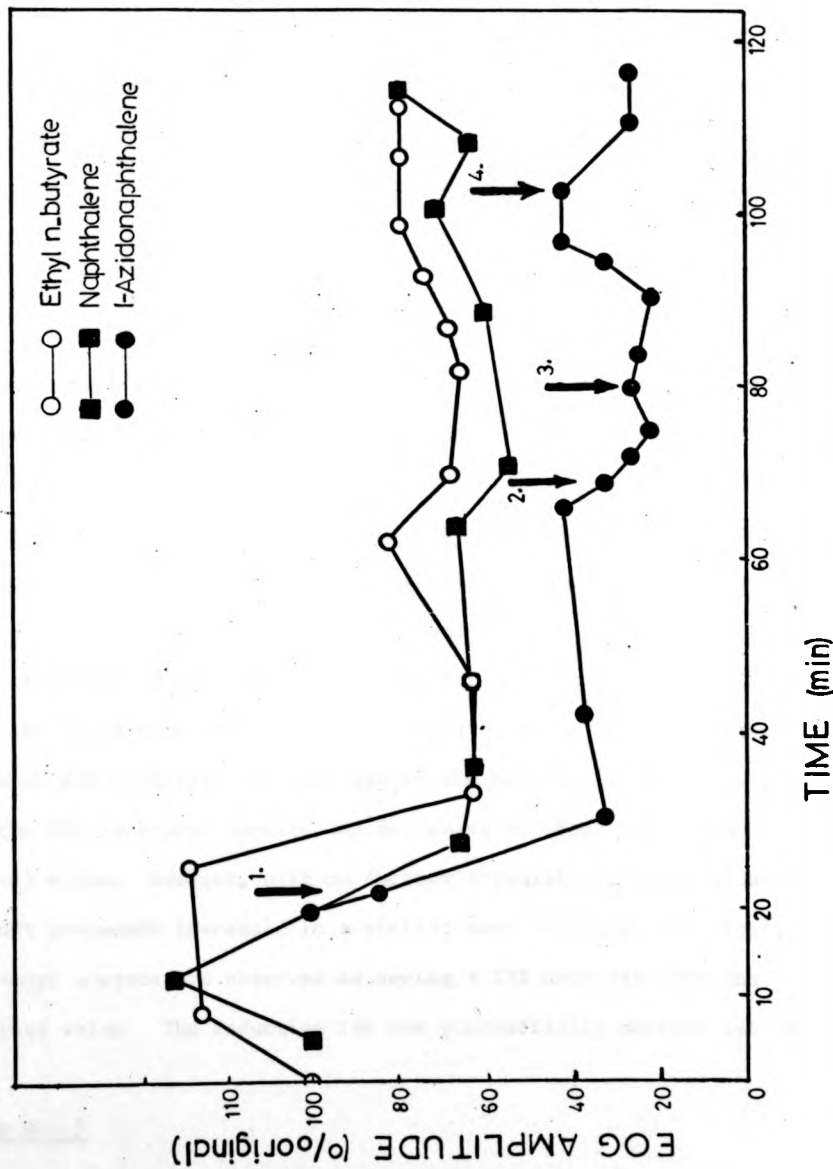
Similar observations have been made about this graph. Here, m-xylene and n-butyl acetate were used as well as naphthalene. The first irradiation on the azide response decreased the EOG by about 10%, whereas the responses to the control odorants increased. However, before the second irradiation it was noticed that a response to the azide also increased. The second irradiation on the EOG to the azide again decreased the response. This was repeated by the third and fourth irradiations, each application being one minute in length, thereby bringing about a 4% decrease in the EOG to naphthalene, 15% to butyl acetate, 30% to m-xylene and 46% to 1-azidonaphthalene at the end of 90 minutes of experimenting.

GRAPH. 1. The effect of light on the responses evoked by 1-azido naphthalene. Irradiations were carried out while the azide was eliciting an EOG response. Duration of irradiations; 1. for 10 sec., 2. for 20 sec., 3. for 40 sec., 4. 30 sec., 5. for 40 sec., 6. for 1 min., 7. for 2 min., 8. for 1 min. Broken arrow (\downarrow) indicates a dark puff of the azide for 1 min.





GRAPH, 2. The effect of light on the responses evoked by 1-azido naphthalene. Duration of irradiations: 1. for 10 sec., 2. for 25 sec., 3. for 1 min., 4. for 1 min.



GRAPH. 3. The effect of light on the responses evoked by 1-azido naphthalene. Irradiations were carried out while the azide was eliciting an EOG response. Durations of irradiations; 1. for 35 sec., 2. for 15 sec., 3. for 20 sec., 4. for 1 min.

Graph No. 3

As is seen after the first irradiation the responses to the azide decreased below 50% value of the control. After the fourth irradiation, the control odorants, ethyl n-butyrate and naphthalene elicited EOGs with only a 21% reduction from their original value, whereas the EOG to the azide decreased by about 84%.

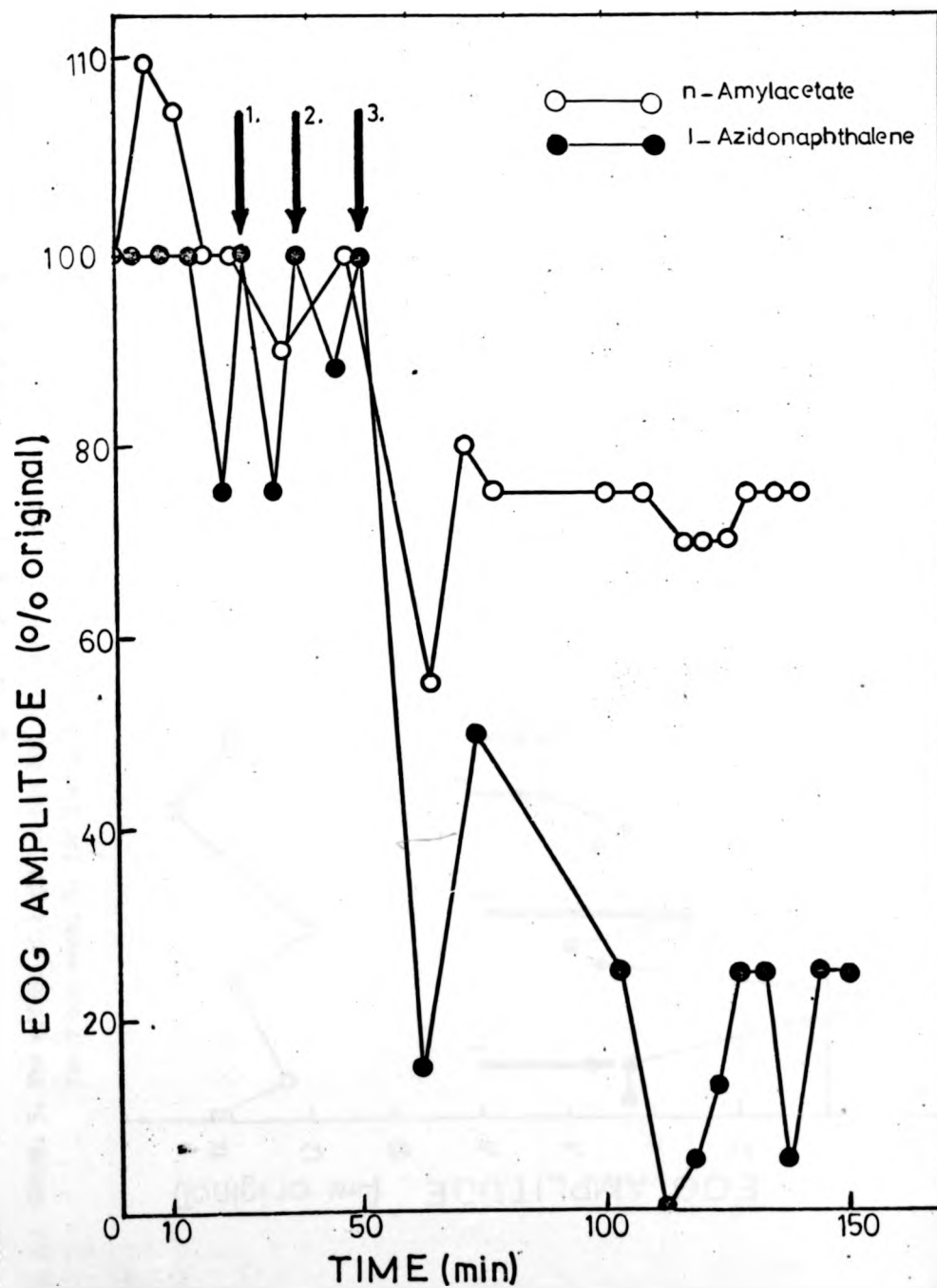
Graph No. 4

This graph gives a much clearer picture than the previous ones. In this work 2 compounds, n-amyl acetate as a control odorant and 1-azidonaphthalene as a photoaffinity odorant, were used. As in the previous experiments, EOG recordings were made from only one area of the olfactory mucosa of the frog (i.e. the electrode position was not changed). The first and second irradiations of 2 minutes duration did not result in any reduction in the EOG amplitude to the azide. With the third irradiation which was applied for a 5 minute period, about an 85% reduction was observed in the EOG amplitude to the azide, and the EOG to n-amyl acetate was decreased by about 45% of the control value. However, with no further irradiation, the responses to both compounds increased in a similar way. Finally, the response to n-amyl acetate was observed as having a 25% decrease from the original value. The reduction for the photoaffinity odorant was 75%.

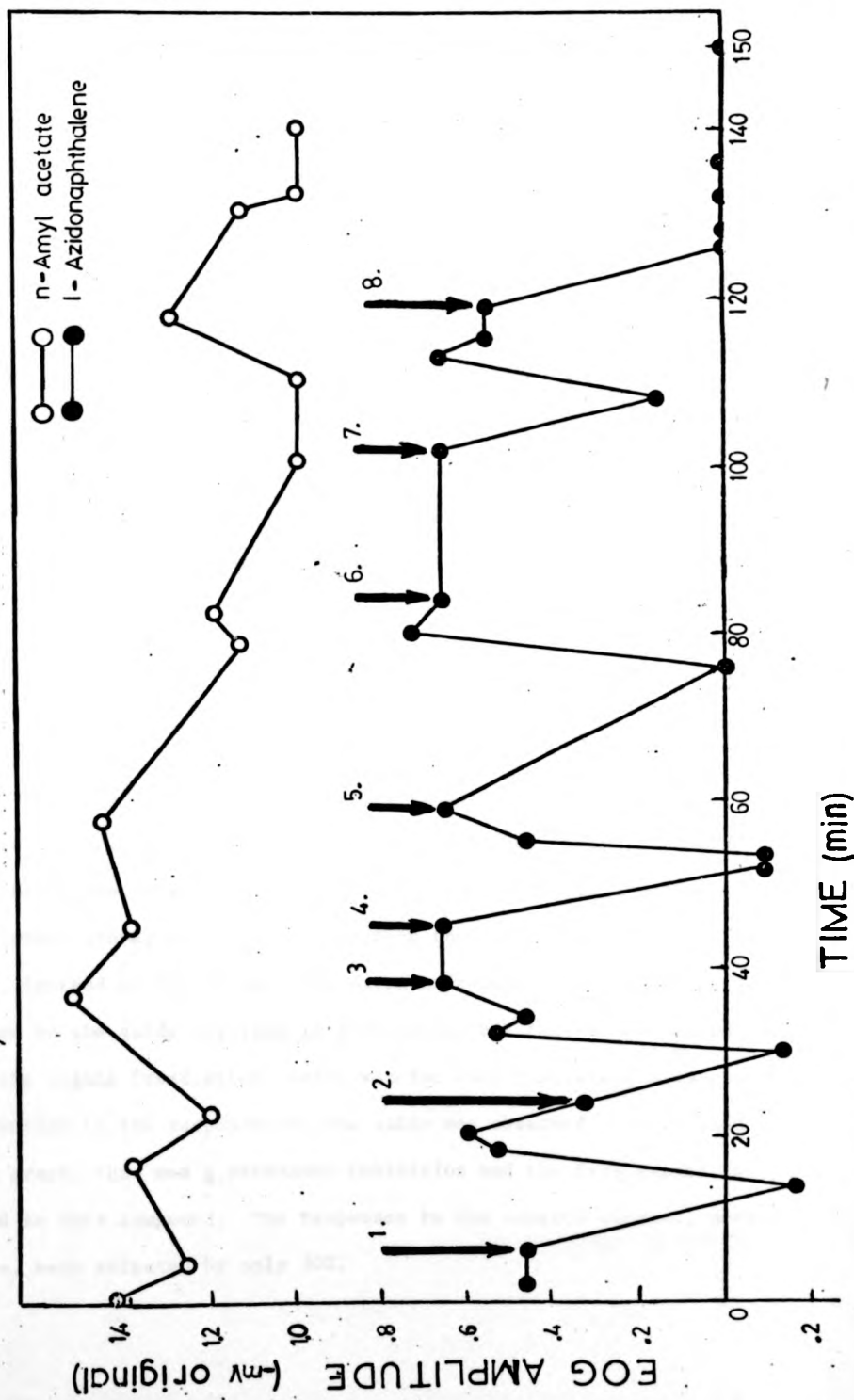
Graph No. 5

This graph is presented to show the plotting of mV amplitude for

GRAPH. 4. The effect of light on the responses evoked by 1-azido naphthalene
Duration of irradiations; 1. for 2 min., 2. for 2 min., 3. for 5min

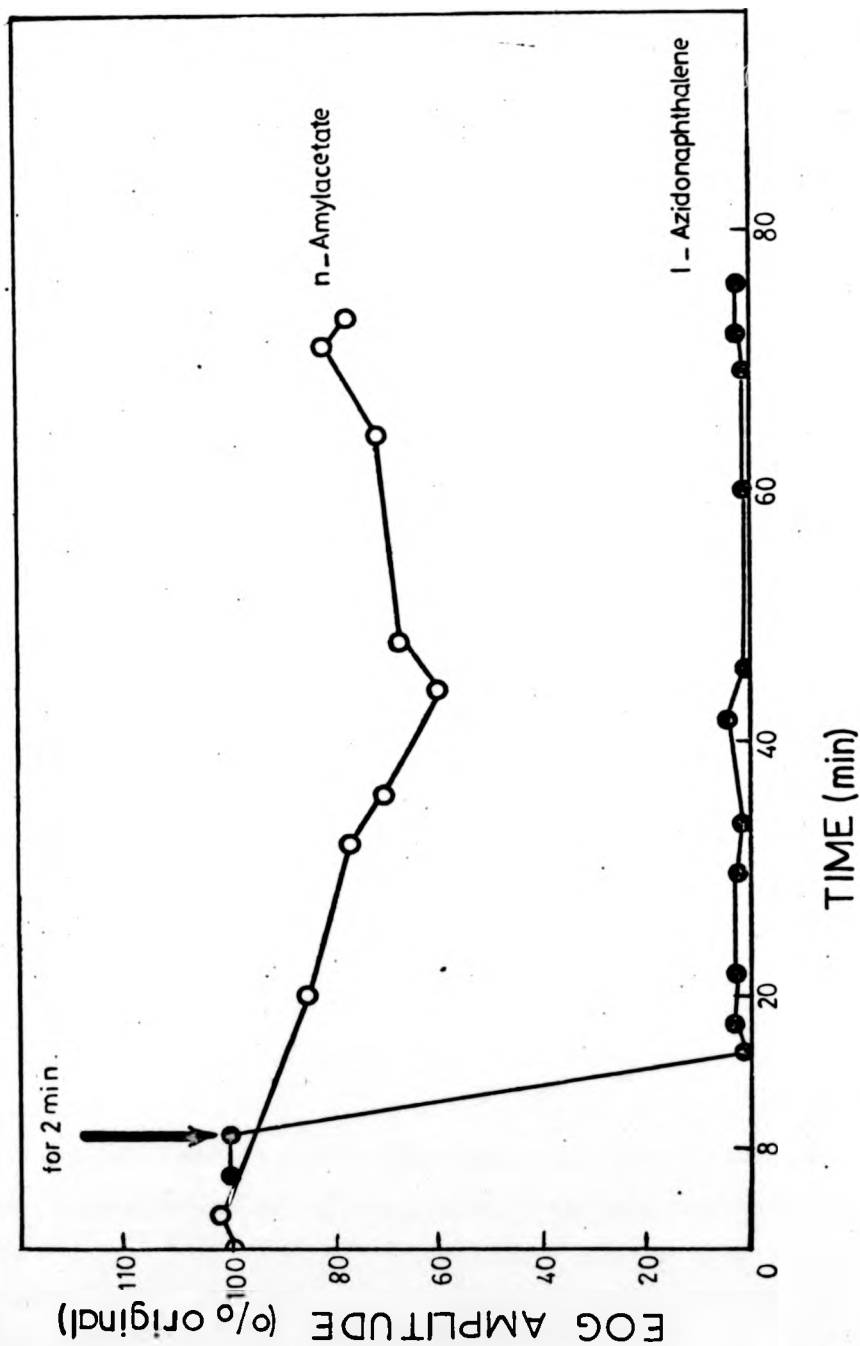


GRAPH. 5. The effect of light on the EOG responses to 1-azido naphthalene. Duration of irradiations; 1-4 irradiations for 1 min each, 5. for 2 min., 6. for 5min., 7. for 1 min., 8. for 2 min.



2 odorants, n-amyl acetate and 1-azidonaphthalene against time. The initial EOG peak amplitudes to the odorants were 1.43 mV and 0.46 mV (n-amyl acetate and 1-azidonaphthalene respectively). With the first irradiation (for 1 minute) a dramatic decrease in the EOG amplitude to the azide was observed. The EOG elicited by the azide after the first 1-minute irradiation was a slow potential in the positive direction (0.16 mV from the resting potential). This inhibition was reversed after a puff of n-amyl acetate. In the second irradiation the above was repeated. But the first two results were not repeated after the third irradiation, after which n-amyl acetate was puffed (this was not the same as in the first two irradiations when the azide was puffed first). Because of this, the inhibition was not seen in the third irradiation. The fourth and fifth irradiations were a repeat of the first two, bringing about reversible blockages in the responses to the azide. With the sixth irradiation, the reversible blockage could not be repeated because, as explained above, after the irradiation the control odorant was puffed, instead of the azide. The seventh irradiation on the EOG response to the azide resulted in a decrease, but this was reversed. After the eighth irradiation, which was for only 2 minutes, a specific inactivation in the responses to the azide was observed. As is seen in the graph, this was a permanent inhibition and the frog ceased to respond to this compound. The responses to the control odorant, n-amyl acetate, were affected by only 30%.

GRAPH. 6. The effect of light on the EOG response elicited by 1-azido naphthalene.



Graph

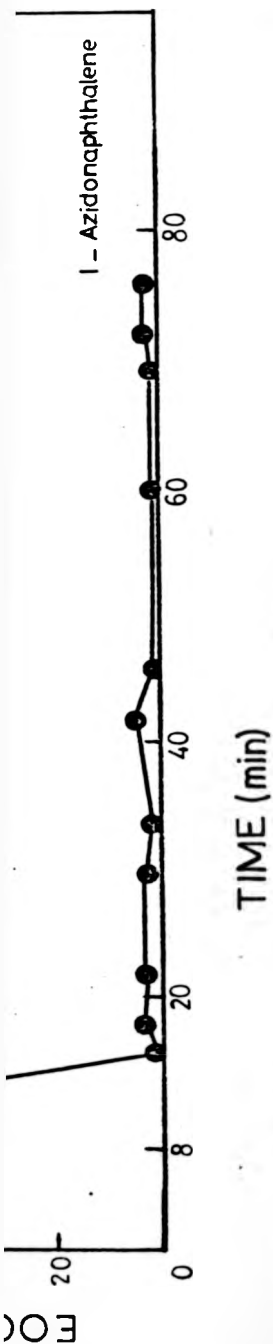
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Graph No. 6

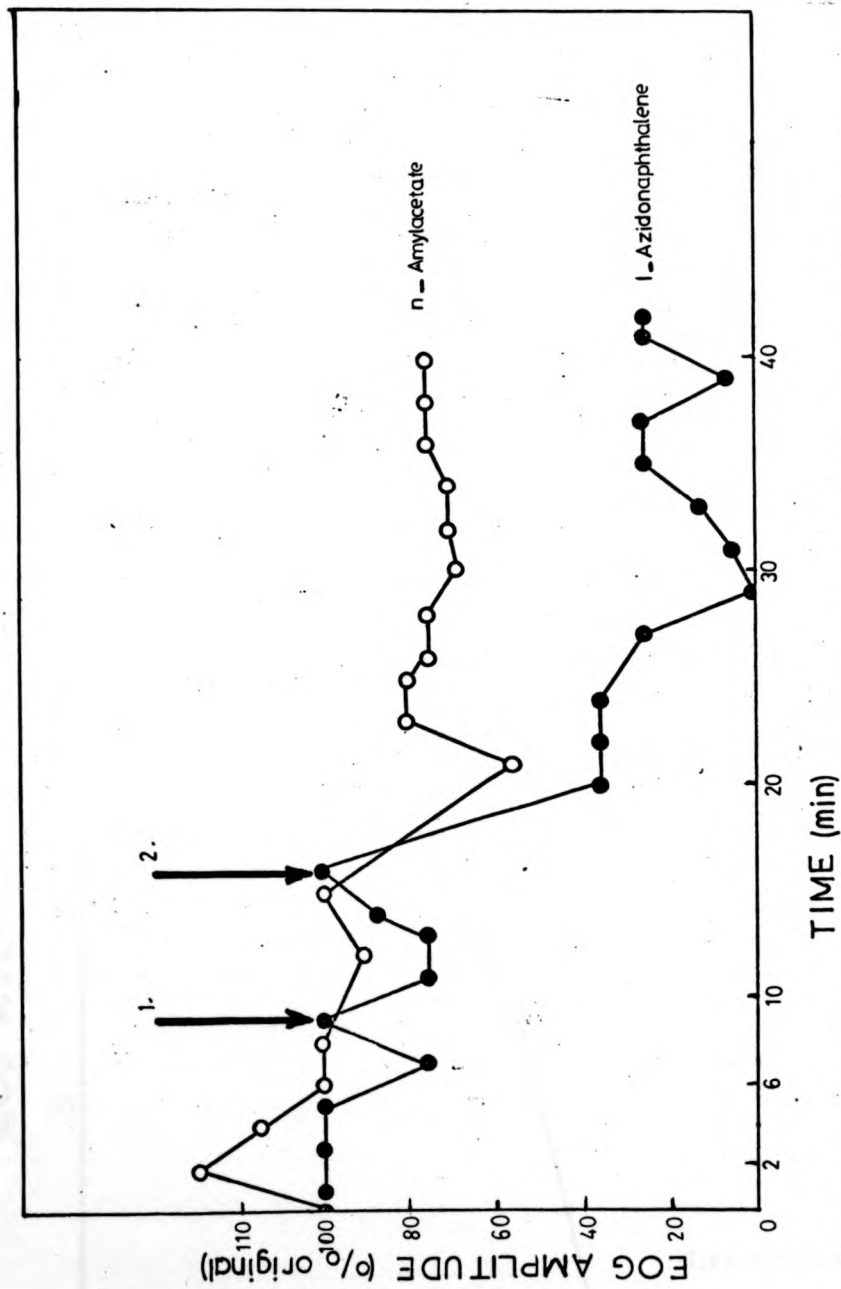
This graph also shows complete inhibition of the responses to the azide but not the control odorant, n-amyl acetate. After the first and only irradiation (for 2 minutes), an irreversible and almost total abolition of the EOG responses to the azide was observed, whilst the EOGs to n-amyl acetate were reduced by only 23%. Responses to the azide remained almost zero throughout the remainder of an hour's recording.

Graph No. 7

This graph shows that during a 42 minute work period, 2 irradiations were carried out on the EOG responses to the azide, of 1 and 2 minutes' duration respectively. After the second irradiation responses to both compounds, n-amyl acetate and 1-azidonaphthalene, were reduced. Again the greater reduction was in the EOG to the azide, which was reduced by about 75% of the original; the control odorant EOG was reduced by only 25%.

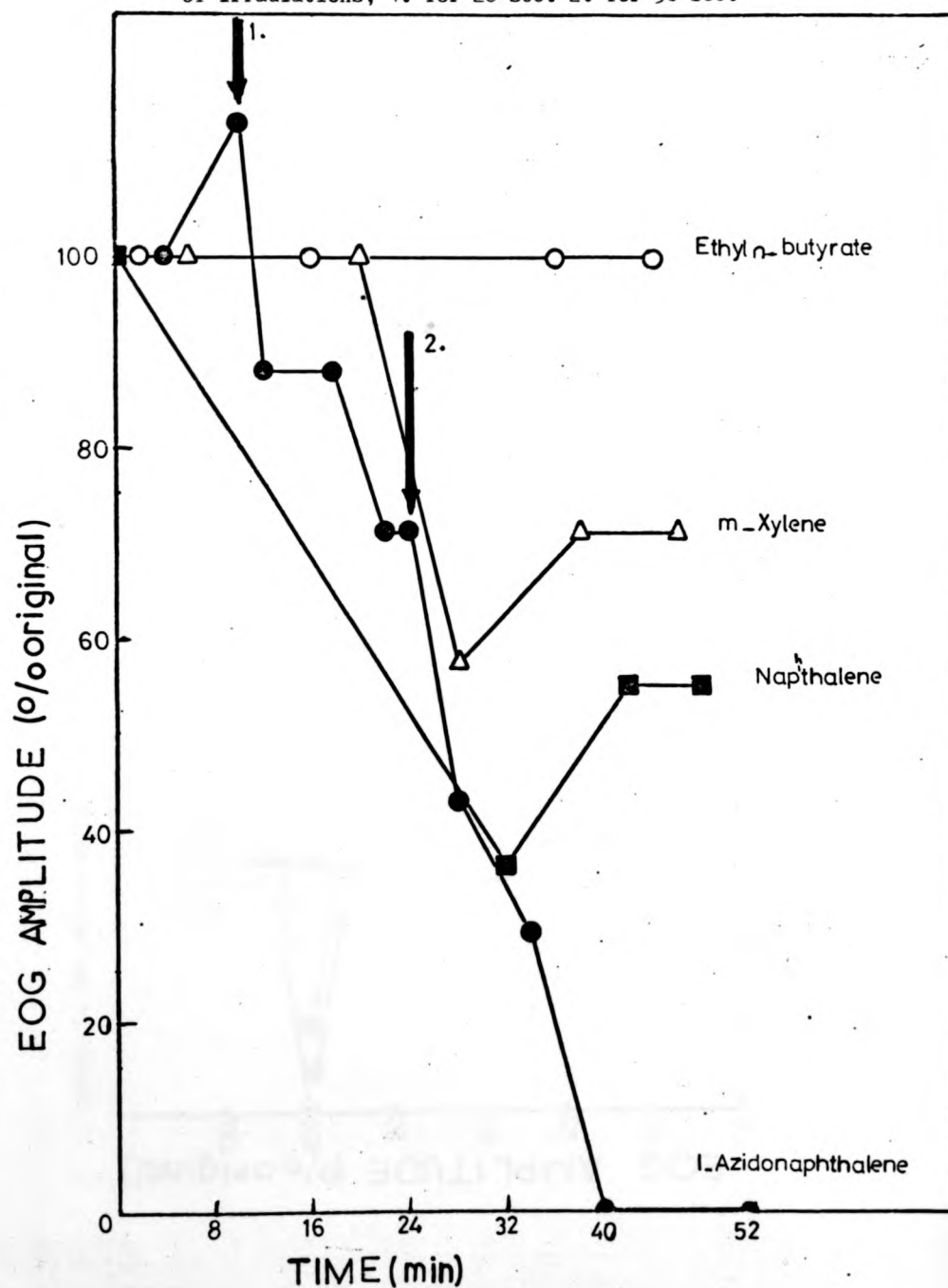
Graph No. 8

Again only two irradiations were carried out with the duration time of 20 seconds and 30 seconds respectively. After the second irradiation the responses to m-xylene and naphthalene were also decreased, along with the reduction of the response to the azide, but

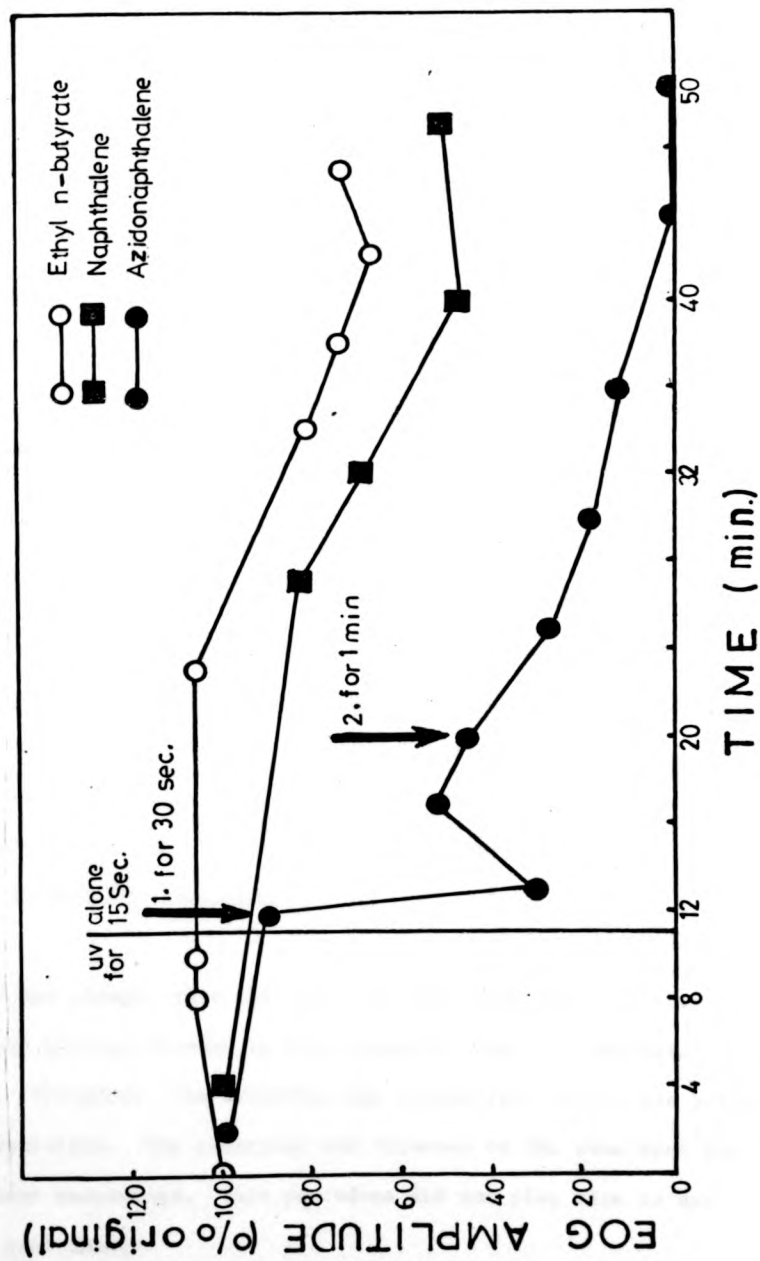


GRAPH. 7. The effect of light on the EOG responses elicited by 1-azido naphthalene. Duration of irradiations; 1. for 1 min., 2. for 2 min.

GRAPH. 8. The effect of light on the EOGs to 1-azido naphthalene. Irradiation were carried out while the azide^{was} eliciting an EOG response. Duration of irradiations; 1. for 20 sec. 2. for 30 sec.



GRAPH. 9. The effect of light on the EOG responses elicited by 1-azido naphthalene.



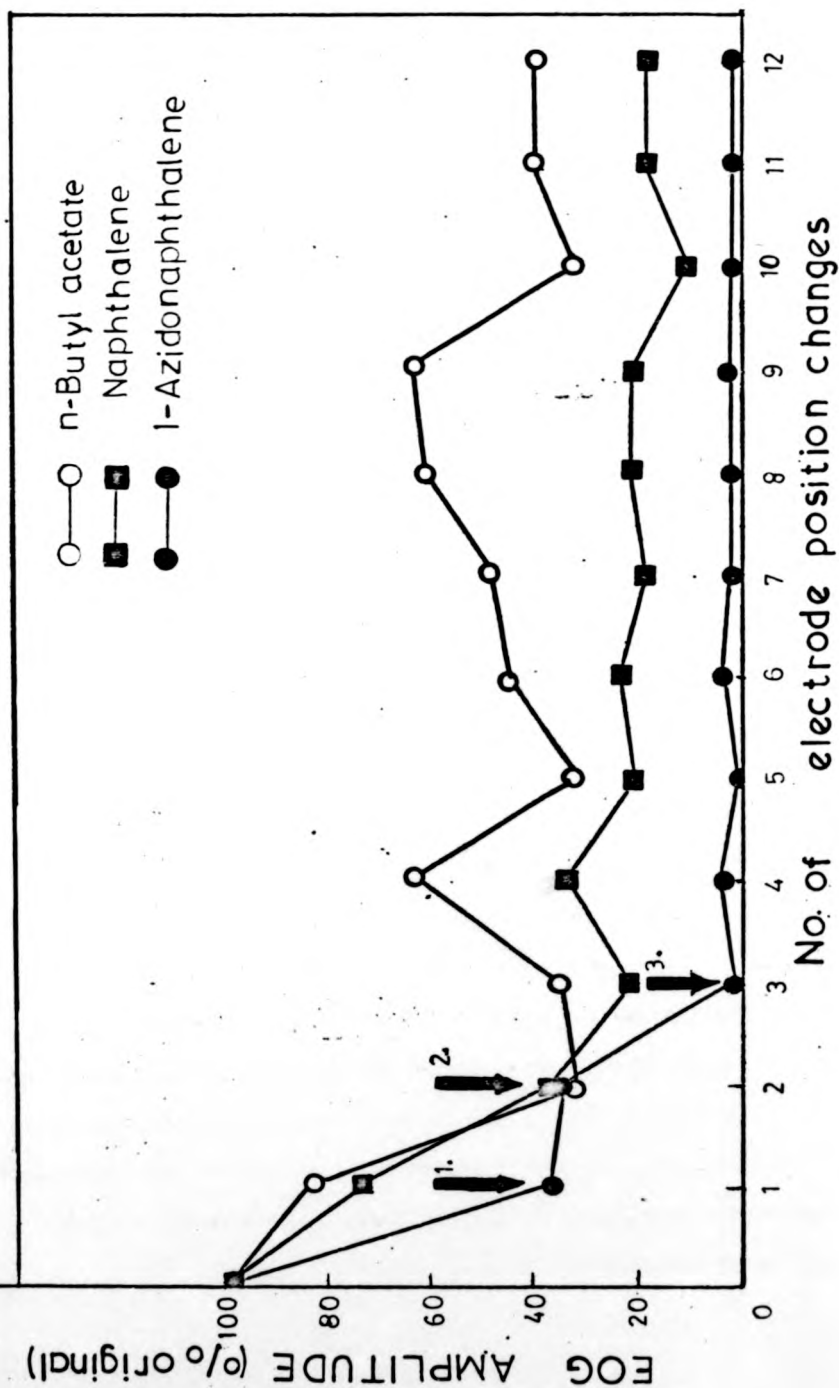
they recovered slightly whereas the responses to the azide continued to decrease and, after 40 minutes, there was no response to the azide. Control odorants had the following EOG value with respect to their original value: m-xylene 71%, ethyl n-butyrate 100%, and naphthalene 55%.

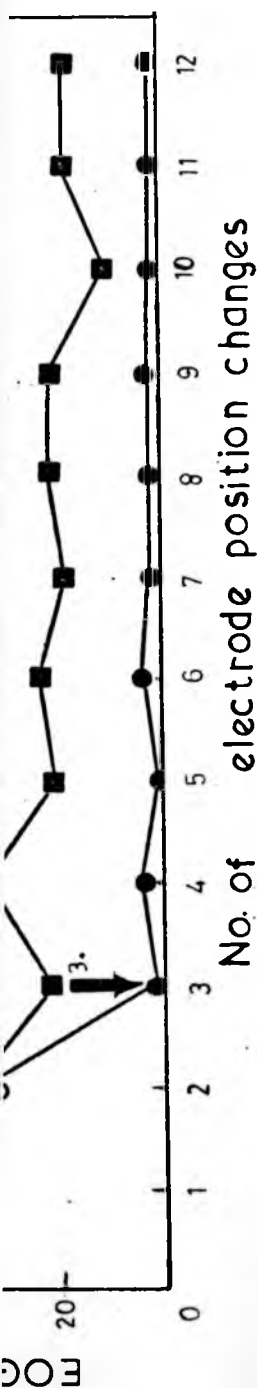
Graph No. 9

Three irradiations were completed with the duration time of 15 seconds, 30 seconds and 1 minute respectively. The first irradiation was the control. The olfactory eminence was exposed to uv light for 15 seconds in the absence of stimuli. The EOG responses to the compounds were not affected. After the second irradiation an irreversible inactivation in the responses to the azide was observed. This was followed by the third irradiation, after which the responses to the azide decreased even more and at the end of 44 minutes, there was no response to the azide. The response to naphthalene was reduced by about 50%. The reduction for n-amyl acetate was only 26% of the control EOG amplitude.

It was thought that the tip of an electrode might be preventing the light getting through to the receptors which are activated by the azides. Therefore, the electrode was lifted from the mucosa before the irradiation. The electrode was returned to the same spot for subsequent recordings. This procedure did not give rise to any significant change.

GRAPH. A. The effect of light on the EOGs to 1-azido naphthalene in different parts of the olfactory mucosa. Duration of irradiations; 1. for 1 min., 2. for 2 min., 3. for 1 min.



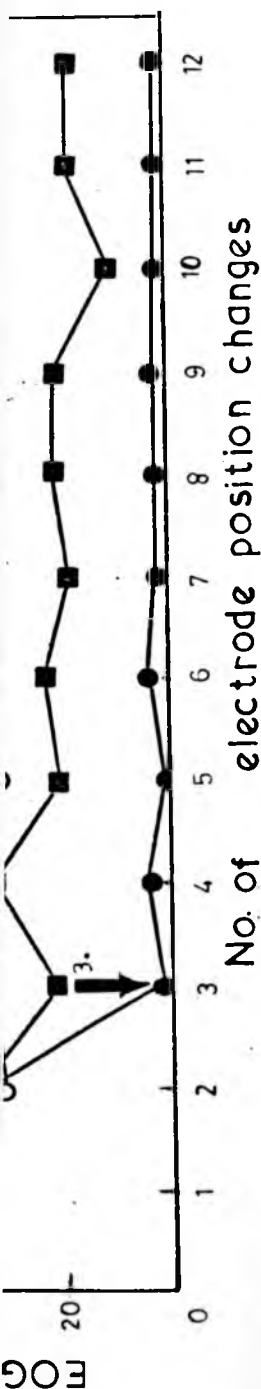


It was also felt that the required intensity of the light might not be reached on every part of the olfactory eminence. Therefore, if one area received the right irradiation, it would result in the blockage of the receptors to the azide in that particular area. Since the receptors to different odorants responded in the same fashion in the same areas (Figure 55), one could find areas from which the control odorants would elicit EOGs but not the azide. Therefore, after a certain time of irradiation of the mucosa in the presence of azide stimuli, the electrode position was changed and EOGs recorded.

Results showed that there were some areas from which recorded responses to the azides were more inhibited than those of the controls after exposure of the eminence to light in the presence of azide molecules. But this is not always so; in a small number of cases, after irradiation, areas were found where relatively larger EOGs in terms of percentage values were recorded for the azides than for the controls.

Graph A shows EOG recordings for 3 odorants; naphthalene, n-butyl acetate and 1-azidonaphthalene, recorded from different parts of the olfactory mucosa of the frog. After three successive irradiations, recordings from 13 different areas showed that the response to the azide was almost abolished, whereas responses to the control odorants, naphthalene and butyl acetate, were reduced by 81% and 60% respectively.

Graph B and C also show that the EOG responses to the azide were more affected than those of the controls in different parts of the olfactory eminence after irradiation of the mucosa.



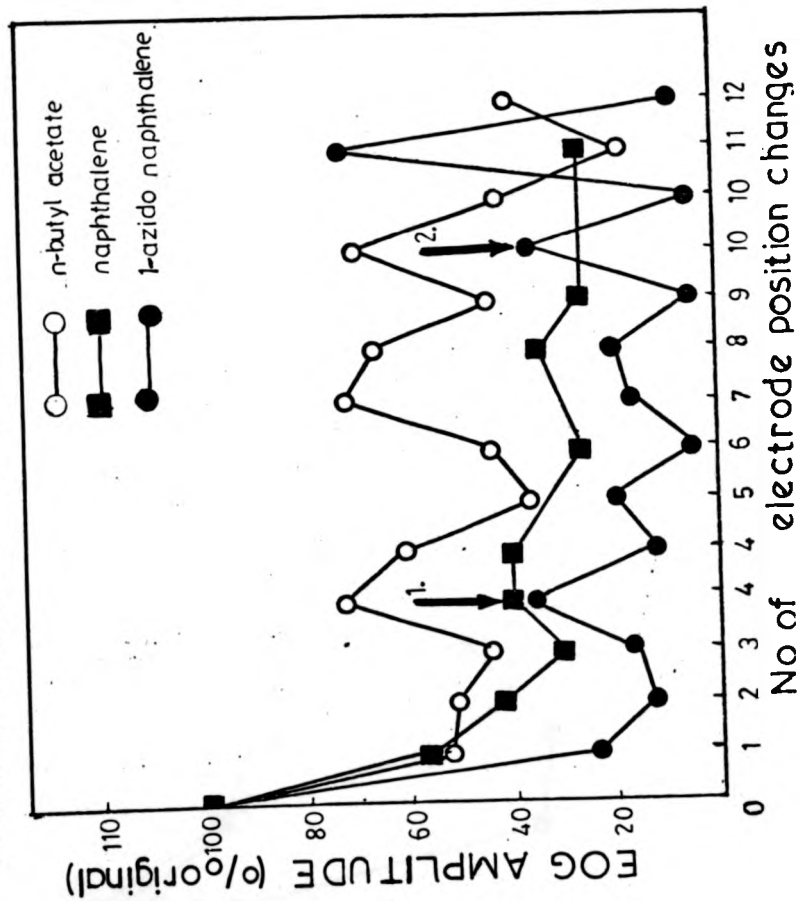
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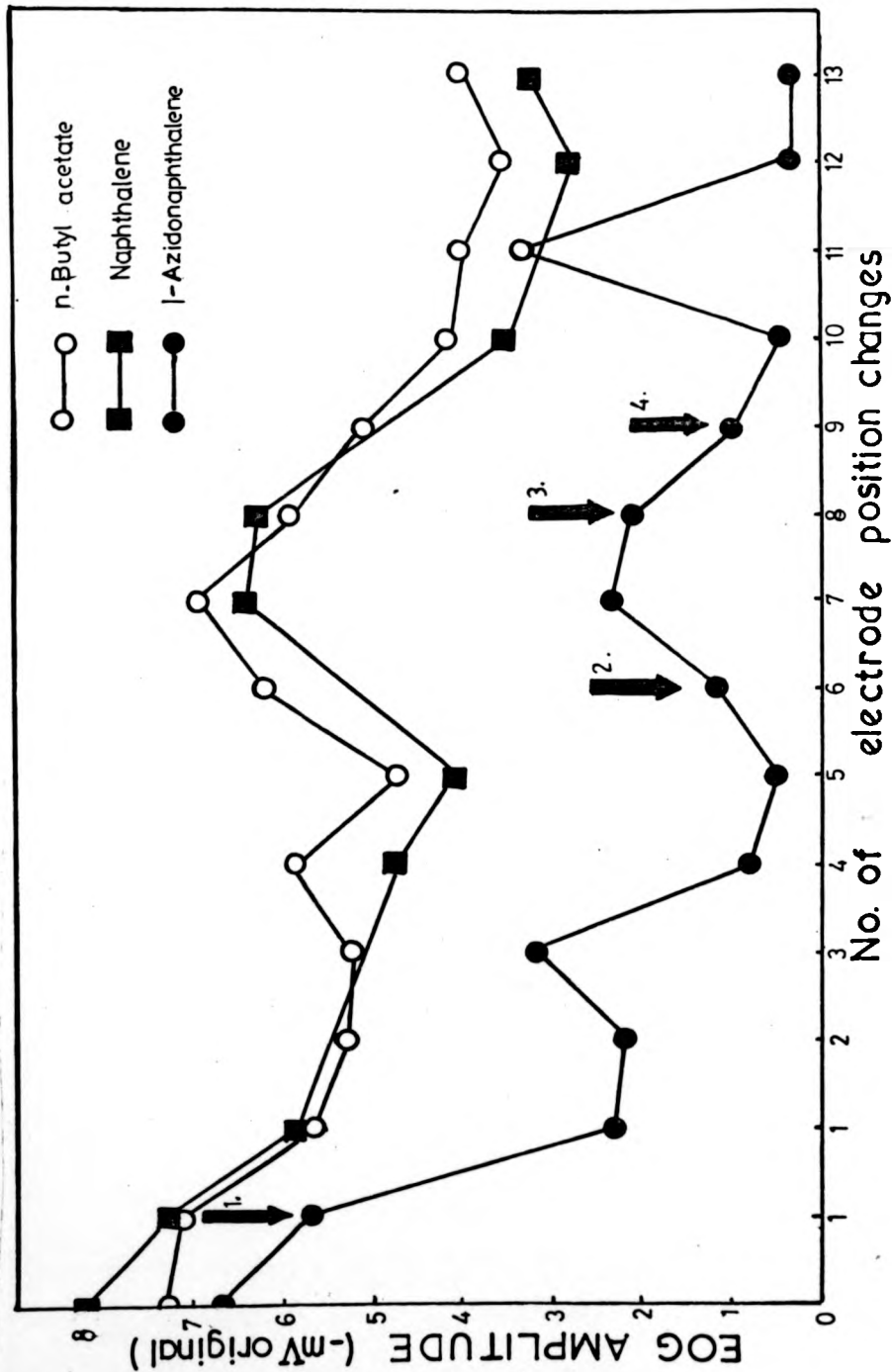
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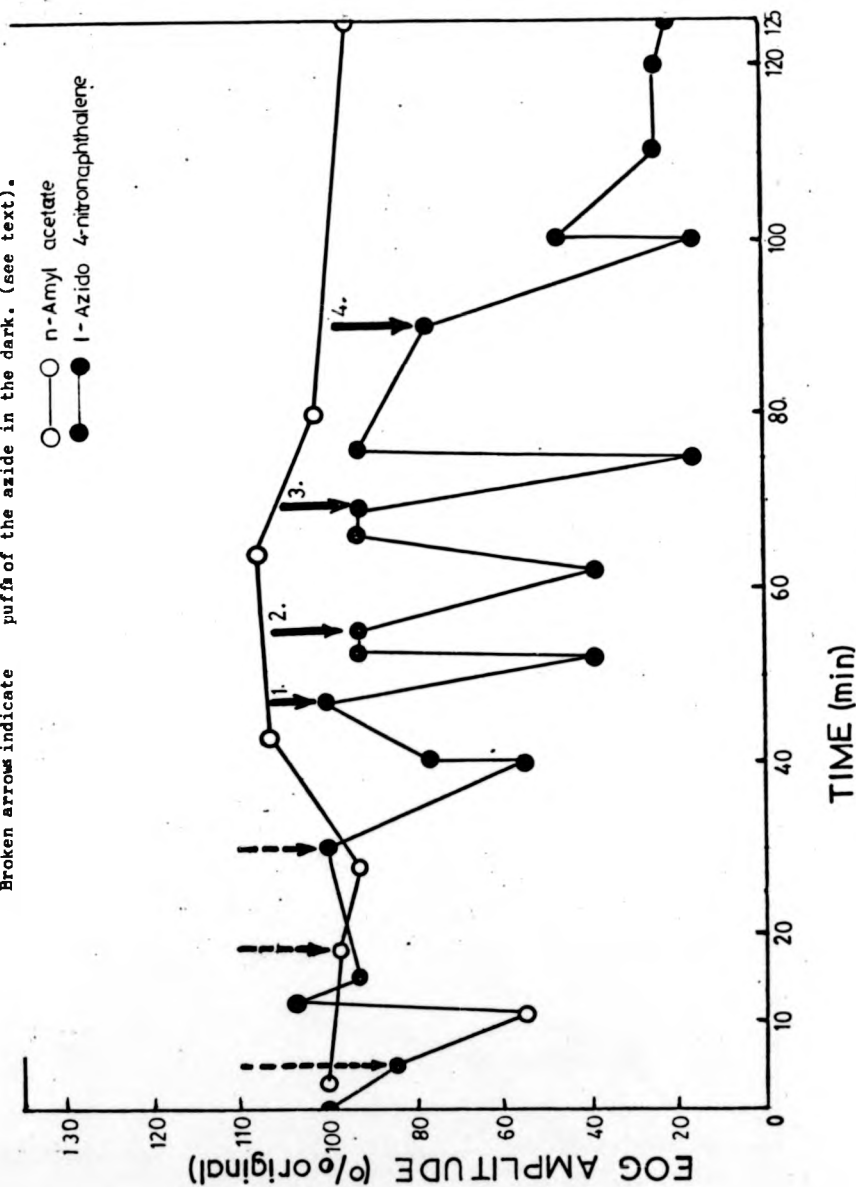
GRAPH. B. The effect of light on the EOGs to 1-azido naphthalene recorded in different parts of the olfactory mucosa. 2 min. durations each.



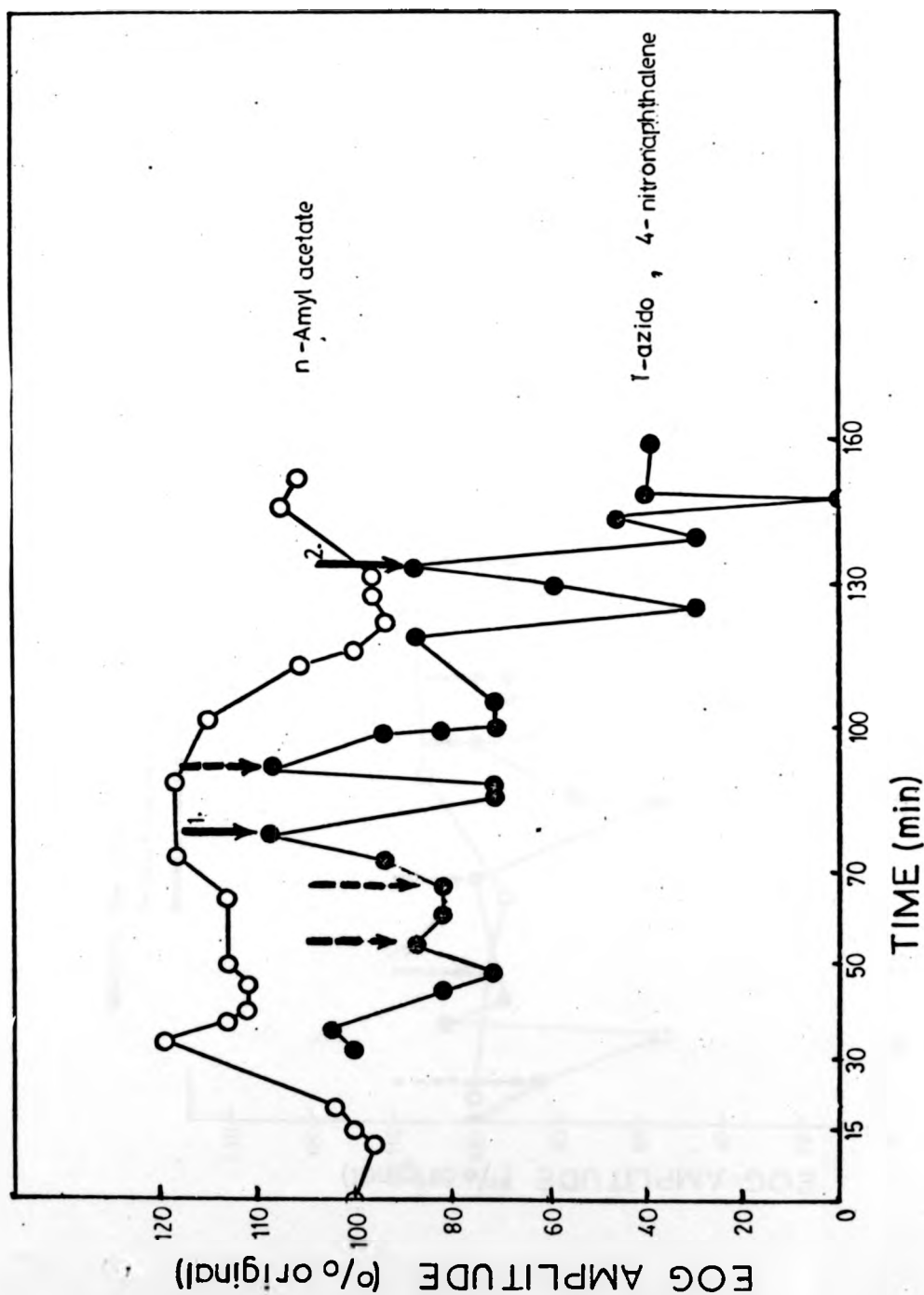
GRAPH. C. The effect of light on the EOG responses elicited by 1-azido naphthalene in different parts of the olfactory mucosa.



GRAPH.10. The effect of light on the EOG responses elicited by 1-azido 4-nitronaphthalene
Irradiations were carried out while the azide eliciting an EOG response.
Broken arrows indicate puffs of the azide in the dark. (see text).



GRAPH. 11. The effect of light on the EOG responses evoked by 1-azido 4-nitronaphthalene

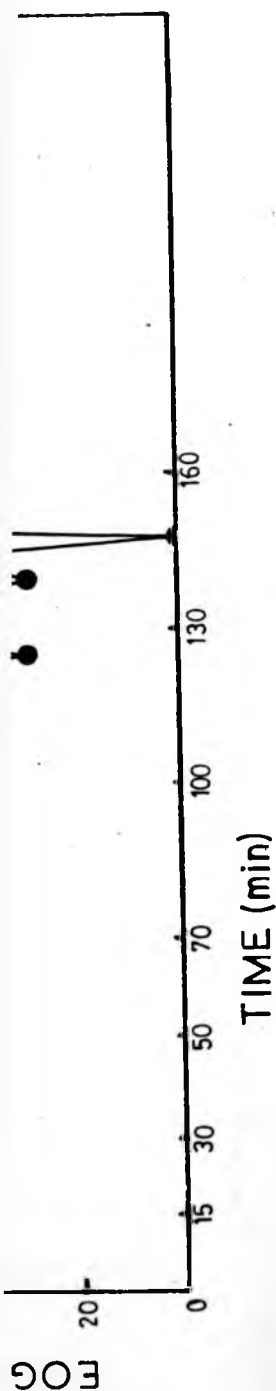


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b) The effect of light on the responses evoked by 1-azido 4-nitronaphthalene

This azide was used as a photoaffinity odorant. It has a spicy smell and elicited normal responses. Although the number of experiments with the use of this azide are few, as some frogs did not elicit EOGs to it; it has again been observed that the EOGs to this azide were reduced upon photolysis, but a complete inactivation in the responses was not achieved.

Graph 10 indicates that exposure of the mucosa to this azide in the dark on two successive occasions, 1 minute and 2 minutes respectively, did not change the response to the azide. Neither did a 2 minute application of n-amyl acetate change the responses to this odorant. The first 3 irradiations, each of 2 minutes duration, gave rise to changes, but they were reversed. With the fourth irradiation which was for 3 minutes, irreversible reduction occurred in the responses to this azide. Finally, at the end of the experiment, the EOG response to this azide was reduced by about 80%, whereas the response to n-amyl acetate was only reduced by 5%.

Graph 11 is somewhat similar to the previous one. It shows that three applications of this azide in the dark, one of 1 minute and two of 2 minutes' duration respectively, did not show any side reaction. With the second irradiation, the responses to the azide started decreasing; this reduction continued until finally it was by about 60% of the original EOG amplitude whereas the response to n-amyl acetate had increased to 110%.

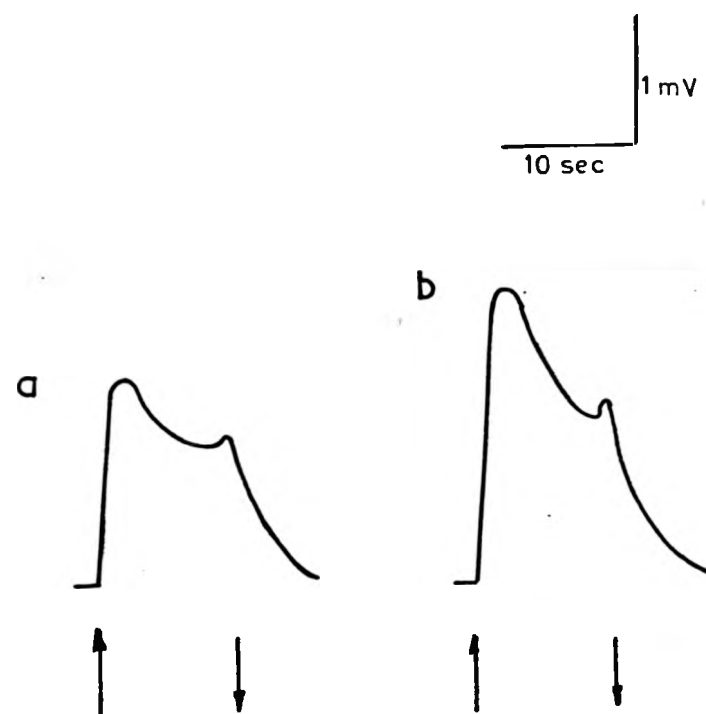


Fig.62. EOG responses elicited by phenylazide (a) and benzaldehyde. (b)

c) The effect of light on the responses elicited by phenylazide

This azide has a similar smell to that of benzaldehyde. It is described as an almond odour (Amoore, 1971). EOGs to this azide and benzaldehyde also showed a great deal of similarity in their EOG shapes. This is especially well-characterised by their "off" response (Figure 62). As is seen in the Figure, this azide behaved normally and elicited EOGs similar to that of n-amyl acetate. However, only 2 frogs out of 6 responded to this azide.

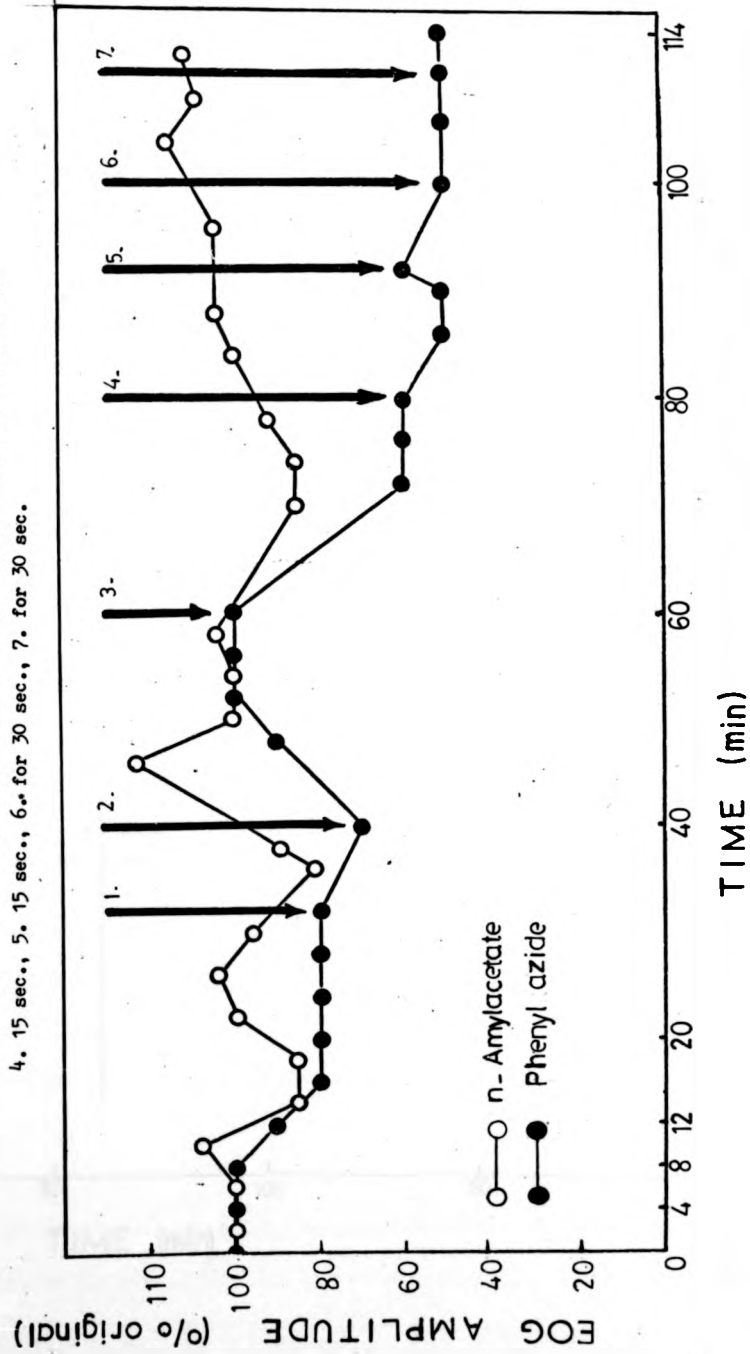
Graphs relating to these 2 frogs showed there was no clear effect of light on the responses elicited by this azide. Although a 50% reduction was observed in the responses to the azide and there was an even increase in the EOG responses to n-amyl acetate, at the end of the experiment (Graph 12), there was a parallel inactivation in the responses to the control odorants, naphthalene and ethyl n-butyrate, as well as phenylazide (Graph 13).

Finally, concerning all azides used in this work, a drop of the azide solution was placed in the centre of a filter paper. This paper was then placed at the same spot where the frog's olfactory mucosa would have been. The filter paper was then irradiated. Within seconds (20 - 40 seconds) a brown colour appeared in the centre of the white paper where the azide had been placed before irradiation.

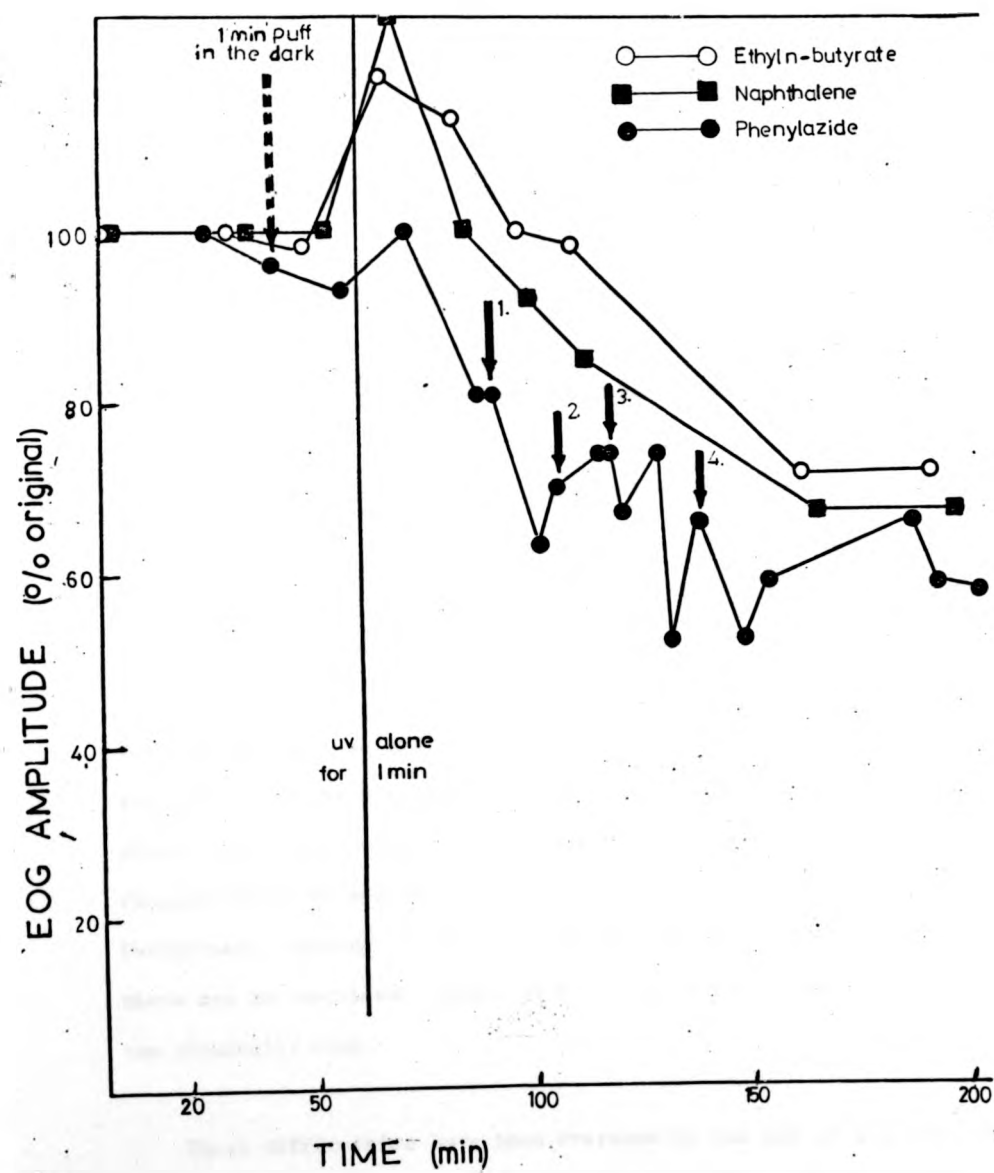
GRAPH. 12. The effect of light on the EOG responses elicited by phenyl azide.

Duration of the irradiations; 1. for 20 sec., 2. for 2 min., 3. for 3 min.,

4. 15 sec., 5. 15 sec., 6. for 30 sec., 7. for 30 sec.



GRAPH. 13. The effect of light on the EOG responses elicited by phenyl azide
 Duration of irradiations; 1. for 45 sec., 2. for 20 sec., 3. for 45 sec.,
 4. for 45 sec.



3.11 DISCUSSION - PART C

The location and identification of drug receptors from complex biological systems with conventional affinity labels are generally limited by the reactivity spectrum of the reagents, which are inadequate for intact receptor systems.

Relating to this, Baker (1967) gave two major reasons: first, the formation of an irreversible complex or covalent linkage to the receptor disrupts the normal receptor function. Although this altered biological response in itself indicates probable site occupation, a reactive affinity label with an organised multi-component system may, in fact, bind too many substituents any of which could interfere with the normal receptor response. Confirmation is needed that the label is capable of interacting at the site of interest and the biological response is generally the only available assay. Second, even if it were assumed that an affinity label could react with a specific receptor, it might simultaneously interact and bind to "non-specific sites" (i.e. spare receptors), so attempts to isolate the true receptor would be complicated by non-specifically bound material. Furthermore, binding to the true receptor may not occur at all if there are no functional groups in the site to which the affinity label can chemically bind.

These difficulties have been overcome by the use of a photoaffinity labelling method (Knowles, 1972). It is, therefore, the technique of photoaffinity labelling rather than conventional affinity labelling

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These difficulties have been overcome by the use of a photoaffinity labelling method (Knowles, 1972). It is, therefore, the technique of photoaffinity labelling rather than conventional affinity labelling

which was applied in this thesis in order to establish the irreversible binding of a photoaffinity odorant (ligand) to its receptor and block the site specifically.

Control experiments relating to the chemical inertness of the aryl azides used in this thesis indicated that they are inert on the EOG responses recorded from the olfactory mucosa in the dark (Figure 54). However, one result came up against this indicating that 2 - 3 minutes' application of 1-azidonaphthalene in the dark reversibly abolished the responses.

Changes in the absorption spectra of the azides after irradiation at a certain wavelength, indicated the generation of a photoproduct (Figure 59). Insertion of this photoproduct, i.e. short-lived intermediate, nitrene into a protein has also indicated that there is a covalent linkage between the reagent and the protein (Figure 59). The finding of this result along with the other publications in the covalent binding process relating to the photoaffinity labelling technique, have suggested that the irradiation of the mucosa with the light during constant stimulation with the azide vapour, may result in inactivation (i.e. insertion of covalent attachment) of the receptors by the azide.

The light intensity of the lamp was also found to be sufficient for the decomposition of the azides. As was described, irradiation of filter paper which had a drop of the azide solution on it changed the colour to dark brown. This is further evidence to the fact that the

azides used in this work are sensitive to the light source used in this work. EOG recordings to the azides have shown that they give different types of EOGs (Figure 54), whereas control odorants always evoked similar types of EOGs. Such discrimination in the EOG responses to the azides may indicate there is a side effect of the azide in the areas concerned with abnormal EOG types. One side effect could be the result of interactions between the azide molecules with different receptors in the membrane. Another side effect may be due to the fact that membrane components involved in ion conductance have been affected by the azides. Thirdly, there may be a dark reaction of the azides. However, mainly the EOG type No. 1, normal EOG, was elicited by the azides.

The effect of light on the responses evoked by the azides clearly showed that the discriminator, i.e. the receptor has been modified. This would mean that either the receptor or the transducer, the linkage between the receptor and the ion conductor, may be affected upon illumination of the frog's olfactory mucosa during stimulation by the azide vapour (Graph No. 6). Therefore it can be assumed that an irreversible reaction (covalent attachment) has occurred between the receptor and the azide.

This type of covalent attachment has also been obtained in neurons. It has been reported (Hucho *et al.*, 1976) that without irradiation the photoaffinity label reversibly inhibited the potassium conductance, whereas the sodium conductance was not affected in myelinated nerve

fibres. With irradiation the potassium conductance was irreversibly inhibited but the sodium conductance was not affected.

In all experiments, it has been noticed that, with irradiation, the reduction in the EOGs to the azides are greater than that to the control odorants. Graphs 5, 6, 8 and 9 show that it has been possible to block selectively and irreversibly the EOG responses to 1-azidonaphthalene. In these experiments the responses to the control odorants were also reduced but by not more than 50% of their control values. Graphs 1, 2, 3, 4 and 7 concerning the 1-azidonaphthalene vapour may indicate that the reduction in the EOGs may be due to covalent attachment between the reagent and receptor. Reductions in the EOGs to the controls may be the result of non-specific binding. This can be explained by the non-specific reaction occurring between the photoproduct of the azide and the other components of the membrane. Such a possibility has recently been observed in sarcoplasmic reticulum membranes. Radioactive 1-azidonaphthalene and 1-azido-4-iodobenzene have been converted into reactive nitrenes which labelled covalently the fatty acyl chains of phospholipids (Klip and Gitler, 1974).

Although the use of specifically designed arylazides as photo-chemical labelling reagents for investigating sites of macromolecules has been an attractive concept for 4 years (Knowles, 1972), recent results in various laboratories have shown that there are severe problems and limitations with the method. The random labelling problem has been observed. This is almost certainly largely due as

In order for true photoaffinity labelling to occur, the rate constant k_2 must be significantly larger than k_{-3} . Under such circumstances, the photolytic intermediate $L^*.P$, which is produced while the molecule $L.P$ is reversibly bound to the active site, will generally react covalently within the site before it can dissociate from it. Those molecules of $L^*.P$ that are produced by photolysis of $L.P$ in free solution may then react with the solvent or with added scavengers, and only minimally with the protein. The specificity of the labelling of the active site in question might thus be very large (Kiefer *et al.*, 1970). On the other hand, if $k_{-3} > k_2$, then pseudo-photoaffinity labelling may result. $L^*.P$ present in free solution may bind reversibly to, and dissociate from, the active site many times before it (or its product) reacts to form a covalent bond within the active site. In principle, therefore, pseudo-photoaffinity labelling is basically the same as conventional affinity labelling.

Aryl azides used as photoaffinity odorants certainly fulfil in part the potential advantages of this approach for the labelling of the receptors as described in the introduction. They do interact with the receptors *in vivo* in olfactory mucosa as evidenced by their EOG waveform and amplitude changes upon photolysis (Figure 61). However, evidence for increased specificity of binding over more conventional approaches is not provided by the observations reported here.

Azides fail to effectively block the receptors which are responsible for initiating the EOG signal. Several explanations are possible. First of all, azides may simply use "common" receptor sites

which may also be used by the control odorants. Reductions on the EOGs to the control odorants may indicate that such non-specific binding could be occurring. Secondly, azides are likely to interact with water around the receptor sites,^{and} such interaction results may physically and unspecifically block different receptor sites. One also cannot eliminate the possibility from the results that they use specific sites as well as common sites. After the blockage of specific sites, common sites may start to become blocked by azides. Graphs 5, 6, 8 and 9 may imply that 1-azidonaphthalene may block its specific sites and may then start blocking common sites used by the control odorants.

A more likely explanation for the failure of a complete blockage in every case is that there may be extensive non-specific labelling. In fact, incorporation of 1-azidonaphthalene into BSA upon photolysis (Figure 59) clearly demonstrates that non-specific labelling can occur. The wide range of reactions available from the reactive nitrene intermediate is an advantage when the photoaffinity labelling reagent is located within the receptor site, but may be a disadvantage otherwise, especially if it reacts with water. The number of nitrenes generated in an experiment is a function of many variables such as depth and amount of mucus, time of irradiation, wavelength, etc.

The results imply that azides interact with more than one receptor, irreversibly inactivating membrane components involved in the EOG responses.

CHAPTER 4

4.1 GENERAL DISCUSSION

Unlike many areas of biochemical interest, the field of olfaction has not had the benefit of a variety of experimental systems available for answering questions about the mechanisms involved. A major quantitative limit that has delayed a direct biochemical approach is a lack of tissue preparations for odorant-receptor binding studies.

There are two main problems in the behaviour of the vertebrate olfactory system. One is the question of the olfactory code and the other is the question of the transduction mechanism.

Both involve lipoprotein components of the neuronal plasma membrane. Attempts to isolate the olfactory receptor cell membranes were continued in this thesis, but failed to yield pure membrane fractions. Therefore, a quantitative study of the two basic problems of olfaction mentioned above could not be achieved.

We are reduced to reliance on an available indicator, the EOG response itself, which is ideally sensitive but limited in specificity in the determination of different odorant-receptor complexes.

A combination of techniques from different disciplines was needed to study the receptors and their physico-chemical environments, which are responsible for the generation of the physiological response, the EOG.

Therefore, I recorded EOGs from the frog's olfactory mucosa to study the coding and transduction mechanisms.

The experiments conducted for the coding have provided evidence that:

- a) the olfactory receptors are proteinaceous in nature, and
- b) that different olfactory receptor proteins exist.

The results indicate the feasibility of studying the olfactory code in the peripheral receptors using chemical modification methods (Menevşe et al., 1976).

Another approach to the existence of olfactory receptor proteins has recently been reported (Menco et al., 1976). With the use of a freeze-etch electron microscopic technique, membrane particles in high density were found in the ciliary membrane of one month old calves but not in the motile respiratory ciliary membrane of the same animal. It was suggested that these particles might be the receptor sites for binding odorants.

Gesteland, (1976) showed the response specificity of individual olfactory neurons. This suggested that these cells are generalists which respond in a differential manner to a wide range of odorants.

Amoore (1974) reported the occurrence of a number of specific anosmias.

All these reports, together with the results obtained in this thesis suggest that there are different types of olfactory receptor proteins.

Kinetic analysis of the protection experiments in which different concentrations of n-amyl acetate were used resulted in the following equivocal picture. For instance, Figure 63 shows the recovery from the effects of 10^{-3} M n-amyl acetate alone. The straight line in the figure shows that the recovery occurs exponentially. Straight lines were also obtained with 10^{-4} mersalyl alone and 10^{-4} M mersalyl plus 10^{-4} M n- amyl acetate (Figure 64). However, when the protection of the receptors by 10^{-3} M and 10^{-2} M n-amyl acetate from the effects of 10^{-4} M mersalyl was analysed in the same way as above, neither lines were found to be straight. This shows that the recovery does not occur exponentially.

One of the basic problems of olfaction which provided the topic for the appendix of this thesis was the study of the transduction mechanism. Results relating to this problem are not clear at this stage for the involvement of cAMP in the production of olfactory generator potentials.

Fig.63 Kinetics of the recovery (r) of the EOG responses
in the protection experiments.

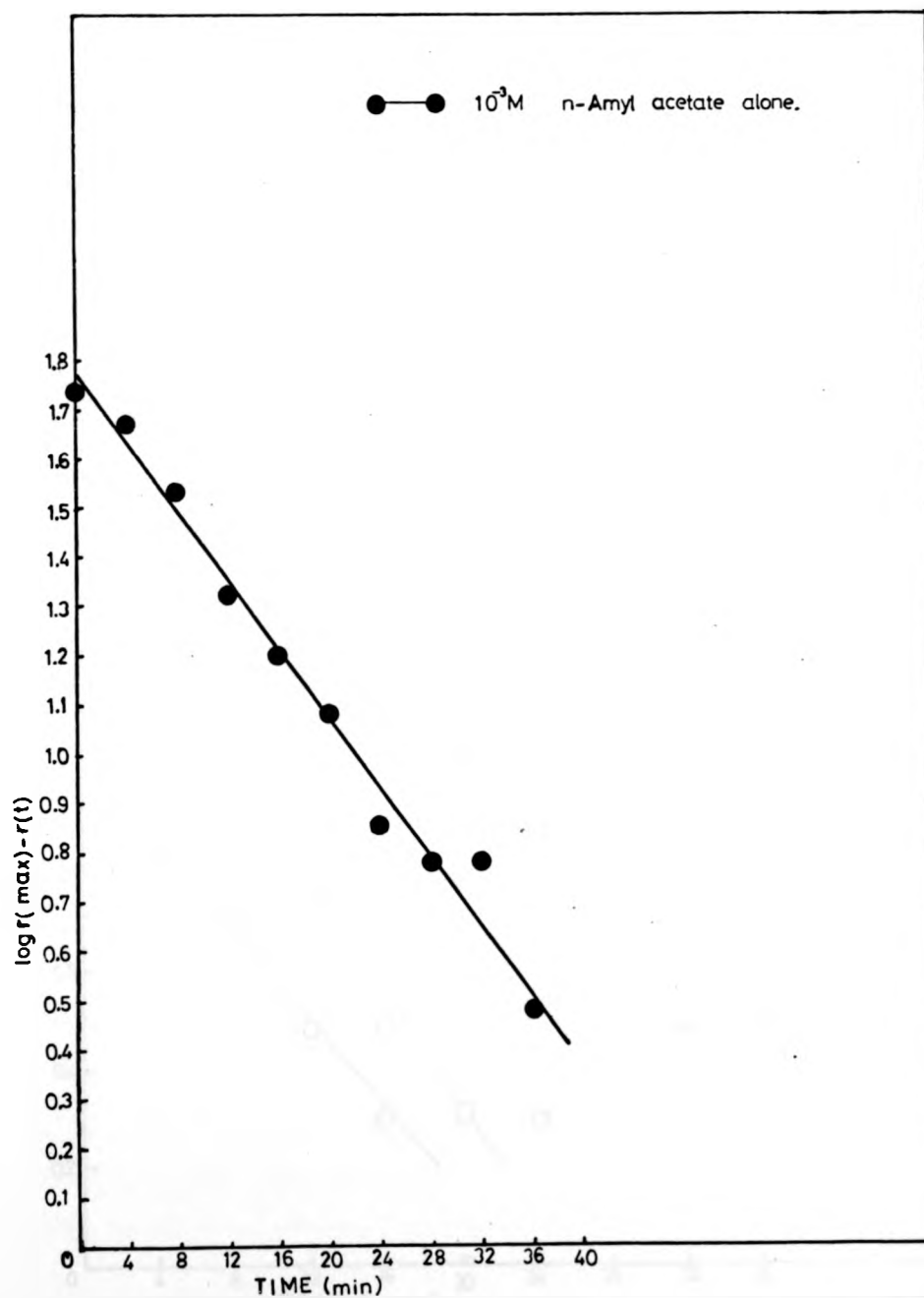
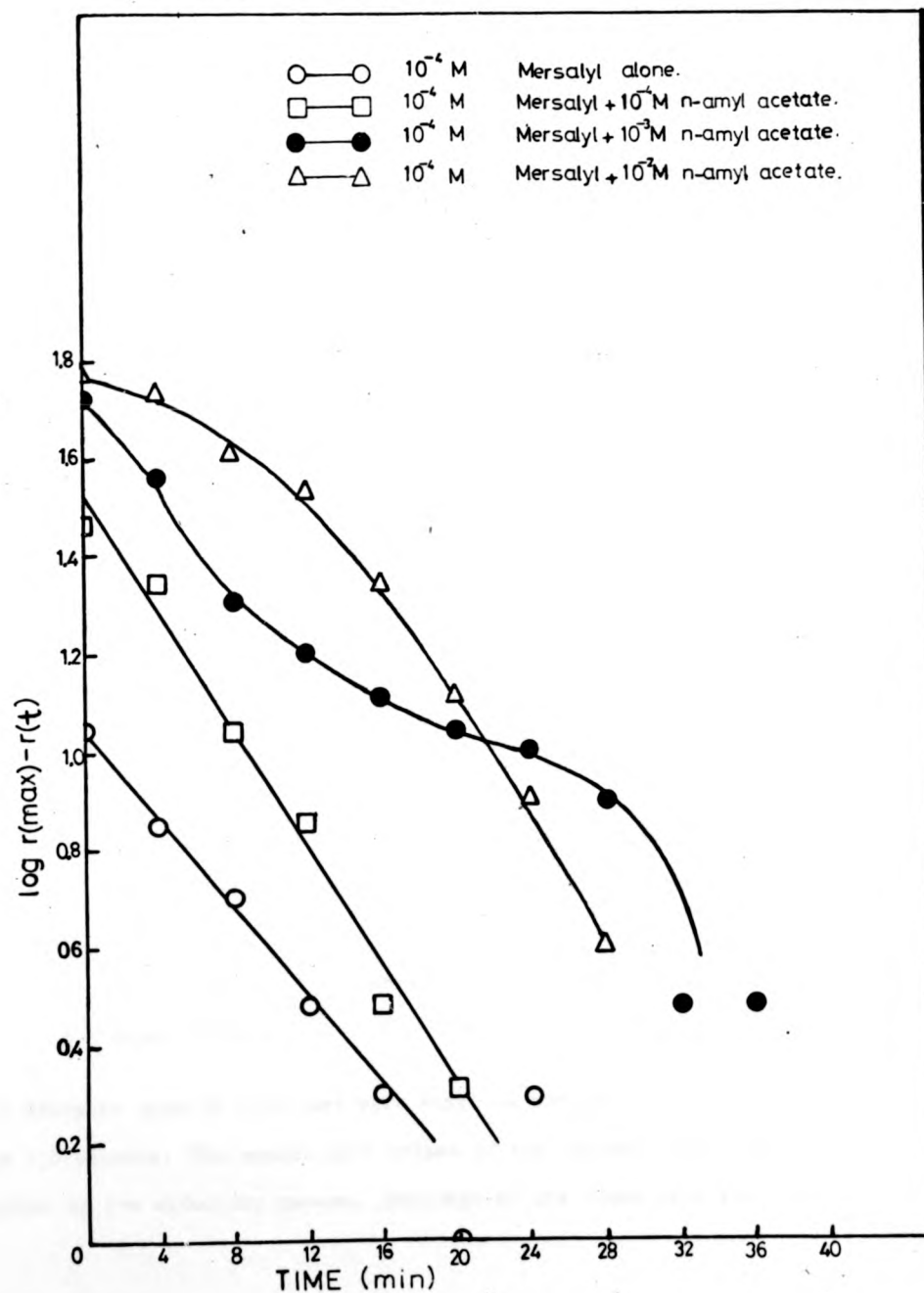


Fig.64. Kinetics of the recovery (r) of the EOG responses in the protection experiments.



APPENDIX

EFFECTS OF OTHER CHEMICALS ON THE EOG RESPONSES

Apart from the protein reagents used in chapter 3, a number of chemicals of possible interest in frog Ringer's solution were applied to the olfactory mucosa. The purpose of the experiments relating to the chemicals used in this part was to conduct a preliminary study on the role of cAMP in the olfactory transduction mechanism. Because, a) the enzyme adenylate cyclase which produces cAMP from ATP was shown to be present in the olfactory mucosa (Menevşe, et al., 1974), and b) the cAMP was shown to be involved in other sensory receptors, namely in visual excitation (Ebrey and Hood, 1973; Miki et al., 1975) in taste reception (Kurihara and Koyama, 1972; Price, 1973), and in neurotransmitter action (Greengard, 1976).

Materials and methods concerning animal preparation, electrodes (EOG recording) and olfactometer have been described in chapter 3.

Chemicals used can be grouped into three classifications.

- 1) Inhibitors of phosphodiesterase (an enzyme hydrolysis cAMP to 5' AMP)
- 2) Cyclic AMP and its membrane-permeable derivatives.
- 3) Other chemicals.

The odorants used in this part were ethyl n- butyrate, n- amylacetate and 1,8 cineole. The square wave pulses of the odorant vapour were applied to the olfactory mucosa. Drainage of the fluid from the cavity

747
was prevented by stopping the buccal aperture with a Teflon strip.

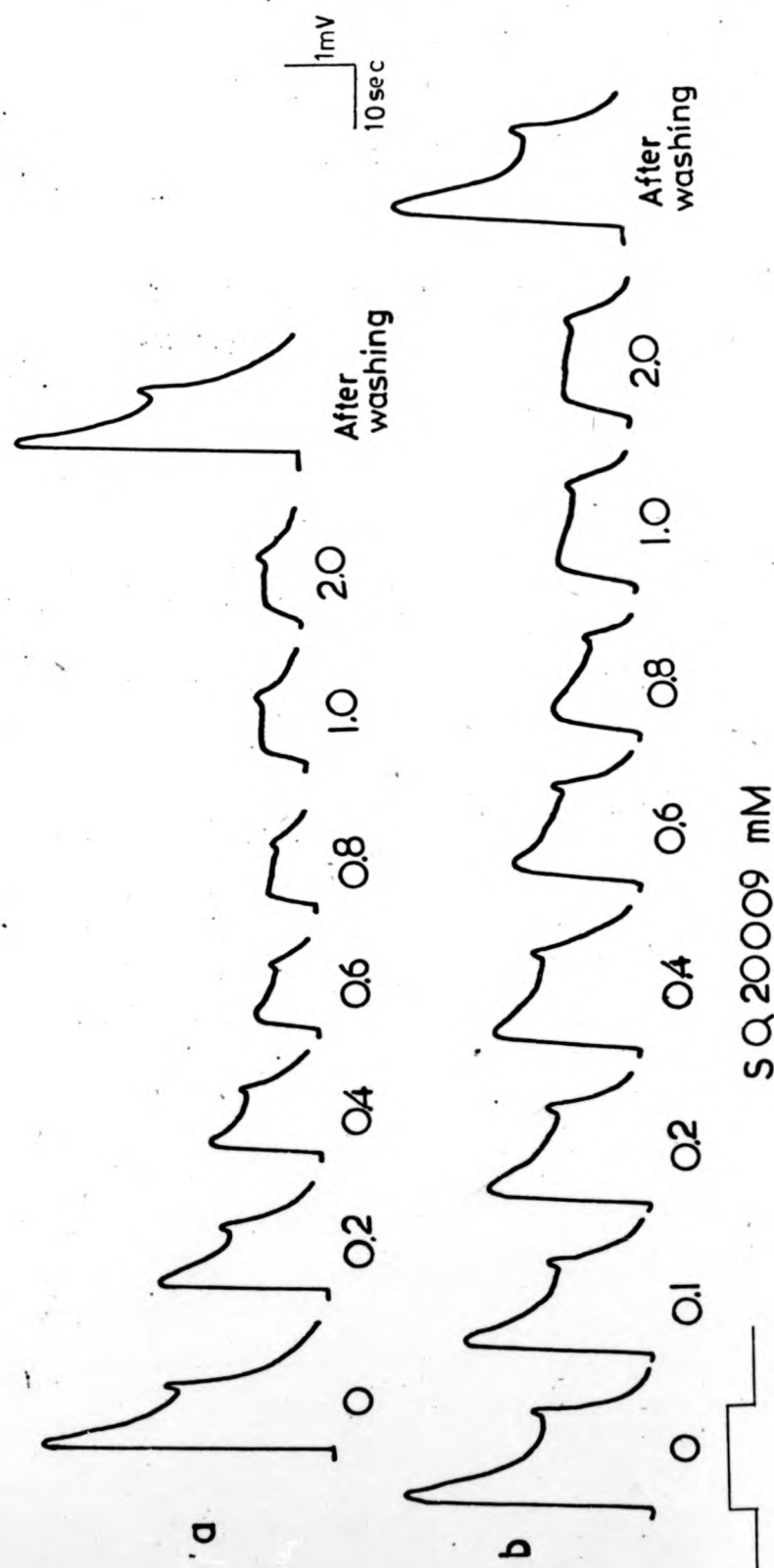
Stimulation procedure

Three test puffs of the stimulus were delivered and EOGs were recorded to determine whether the receptors were responding normally. All stimulations were between 10 to 15 seconds in duration (precise values are shown in each figure), and an interval of 2 minutes was allowed for recovery between each stimulation. After delivery of the test puffs, the recording electrode was lifted by means of a micro-manipulator, and its tip placed in a beaker of Ringer's. The olfactory cavity was filled with Ringer's solution. One c.c. disposable syringes with stainless steel needles were used to fill and drain the olfactory chamber. Separate syringes and needles for filling and draining were used for each solution. Solutions were gently introduced into and withdrawn from the cavity with the needle to one side of the olfactory eminence so as to avoid mechanical damage to the tissue. The Ringer's solution was allowed to remain in the cavity for 10 minutes. Three solution changes were made during this 10 minute period as a control for washing, one immediately after filling and the others at the end of every 5 minutes. After withdrawal of the physiological solution, the recording electrode was lowered to the same spot as for the test puffs, and three EOGs were elicited. These responses were used as the control responses (an average of these three). The micropipette was again lifted and its tip was placed in Ringer's.

The olfactory cavity was then filled with a solution of the drug

in Ringer's and the solution was left on the mucosa for one to three minutes, then withdrawn, and one EOG was elicited. This procedure was repeated until the response was abolished or reached a limiting value. In this way, the time course of development of the effects of the drug could be followed. Also the effect of the drugs on the EOG amplitude was carried out by titrating the mucosa with increasing concentrations of the drug. Again after every application of the drug at a particular concentration the solution was withdrawn and the EOG was elicited. At the end of the highest concentration of the drug used, the solution was withdrawn and the EOG to a certain odour was elicited. In this way, the effects of the drug were determined. To test the degree of reversibility of the effects of the drug, the electrode was lifted and the cavity was again filled with the Ringer's for 10 minutes with the same pattern of solution changes as described above. The cavity was then drained, the electrode was lowered and three EOGs were elicited.

Fig.65. The effect of SQ 20009 on the EOGs to a) n-amyl acetate and
b) ethyl nbutyrate (2min. exposure duration)



1) EFFECTS OF PHOSPHODIESTERASE INHIBITORS

Four different phosphodiesterase inhibitors, theophylline, caffeine, RO 20-1724 and SQ20009 were used. The main effect of these inhibitors was found to be on the EOG peak amplitude. They all caused similar changes in the EOGs by first decreasing and then abolishing the EOG peak amplitude. Figure 65 shows a typical effect of SQ20009 on the EOG amplitudes elicited by two floral odorants, ethyl n-butyrate and n-amyl acetate. As is seen the peak component of the EOG amplitude decreases with increasing concentration of the drug. The plateau component of the EOG was also decreased. This is clearly observed with the use of SQ20009. Figure 66 shows that the drug reduced the plateau component of the EOG as well as the peak component. However, in the case of the inhibitors, theophylline and RO 20-1724, such an effect was not observed until the EOG peak was abolished. Figure 67 shows the effect of theophylline on the peak and plateau amplitudes of the EOG. A typical result concerning the effect of theophylline on the EOG responses to n-amyl acetate is shown in Figure 68. It can be seen that the plateau component of the EOG was increased after 1mM application of the drug for 2 minutes. The "off" responses of the EOGs were not affected by the inhibitors (Figure 68). The EOG amplitude decreases with increasing concentration of the inhibitors but is not abolished at the highest inhibitor concentration used; instead the curves exhibit a maximum value of inhibition which is characteristic for each inhibitor (Figures 69a, b, c, d and e).

After
washing

2.0

1.0

0.8

0.6

0.4

0.2

0.1

0

SQ20009 mM

Fig.66. The effect of SQ 20009 on the EOG peak and plateau components of n-amyl acetate (1min application of each conc., mean \pm SEM. of 6 exp.).

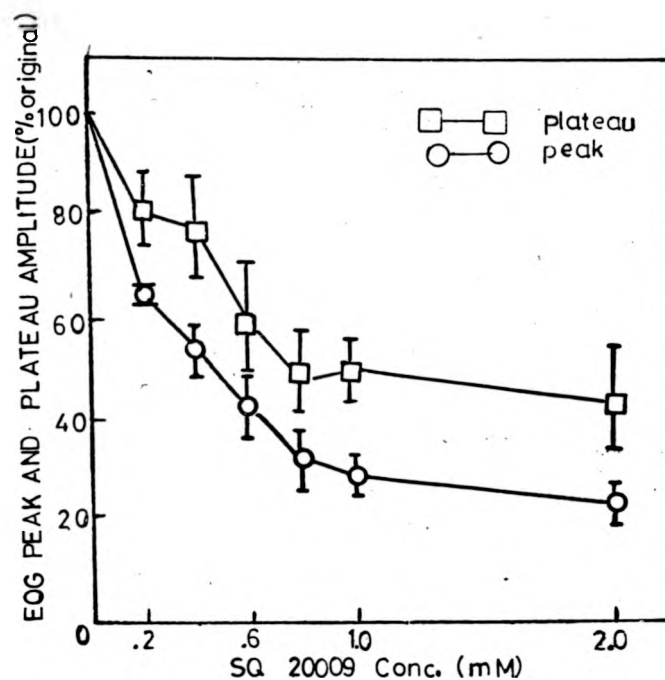


Fig.67. The effect of theophylline on the EOG peak & plateau components of n-amyl acetate (2min application of each conc., mean \pm SEM. of 3 exp.).

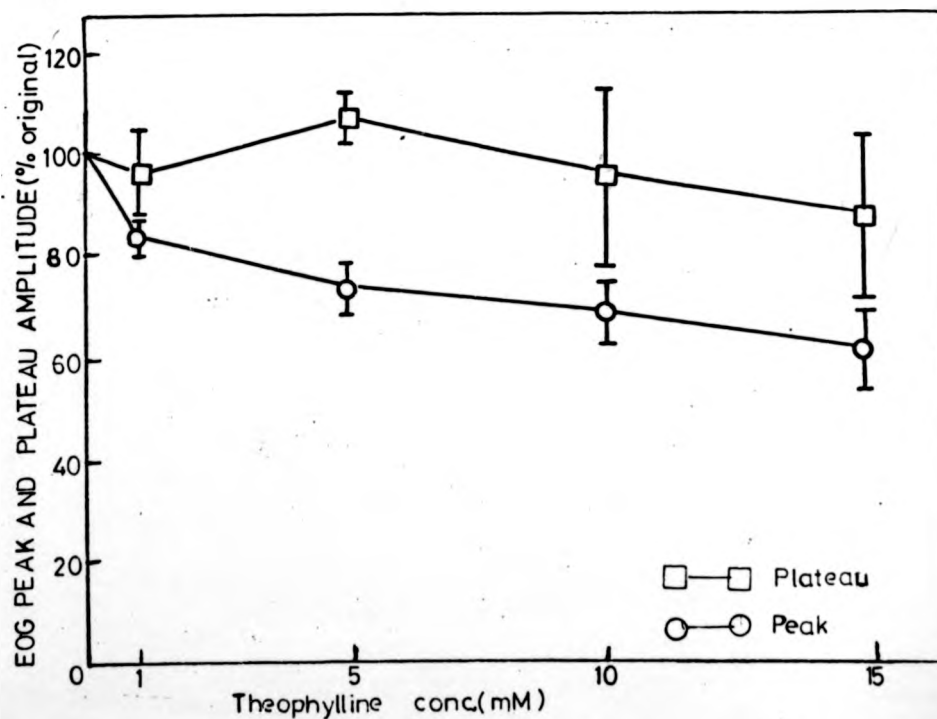


Fig. 68. The effect of theophylline on the EOGs to n-amy acetate (2 min application of each conc.)

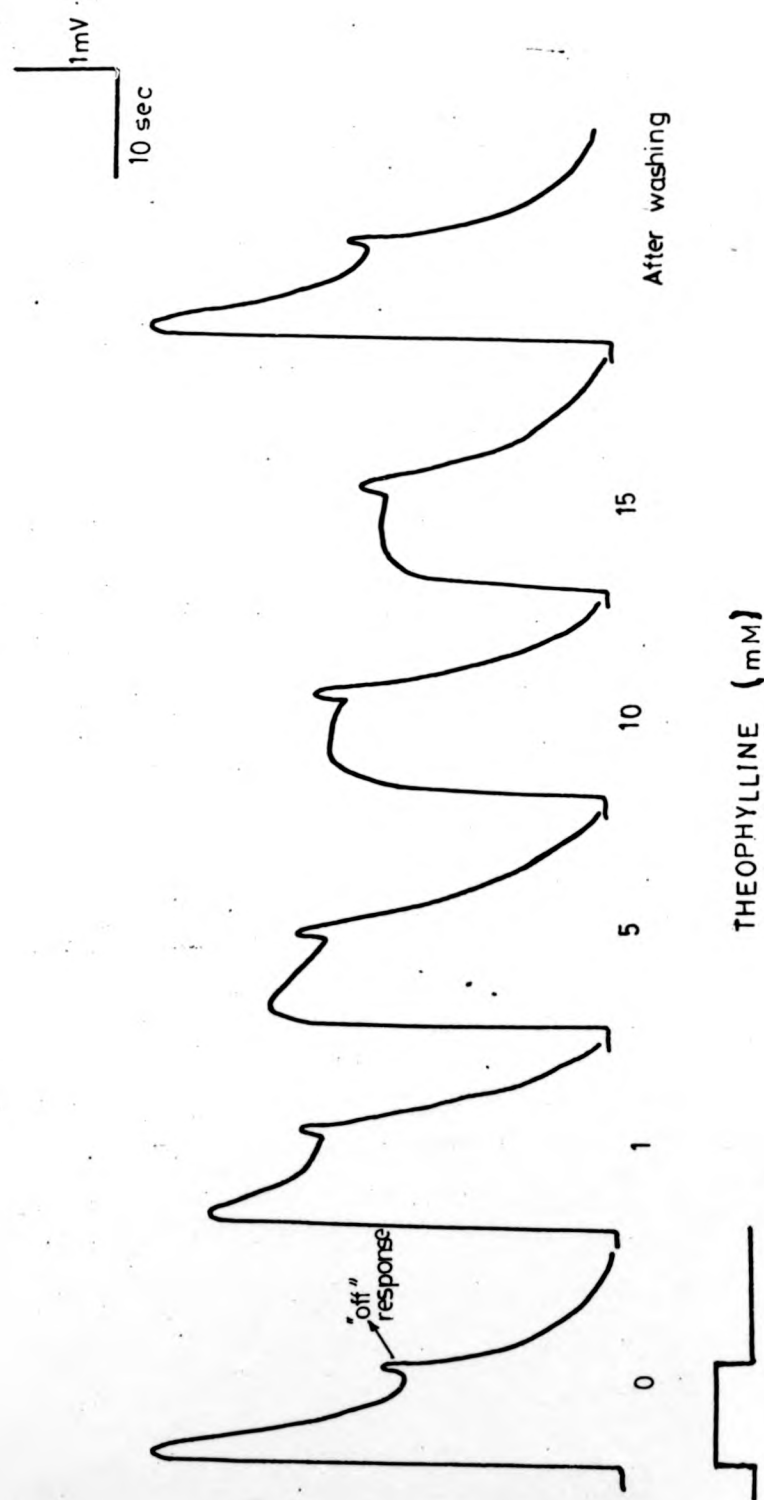


Fig. 69a.

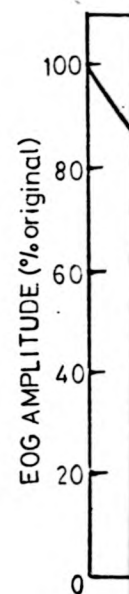


Fig. 69b



Fig. 69a. The effect of theophylline on the EOGs to n-amyl acetate. The broken lines indicate the recovery of the responses after the washing procedure. (2 minutes application of each conc., mean \pm S.E.M. of 3 exp.)

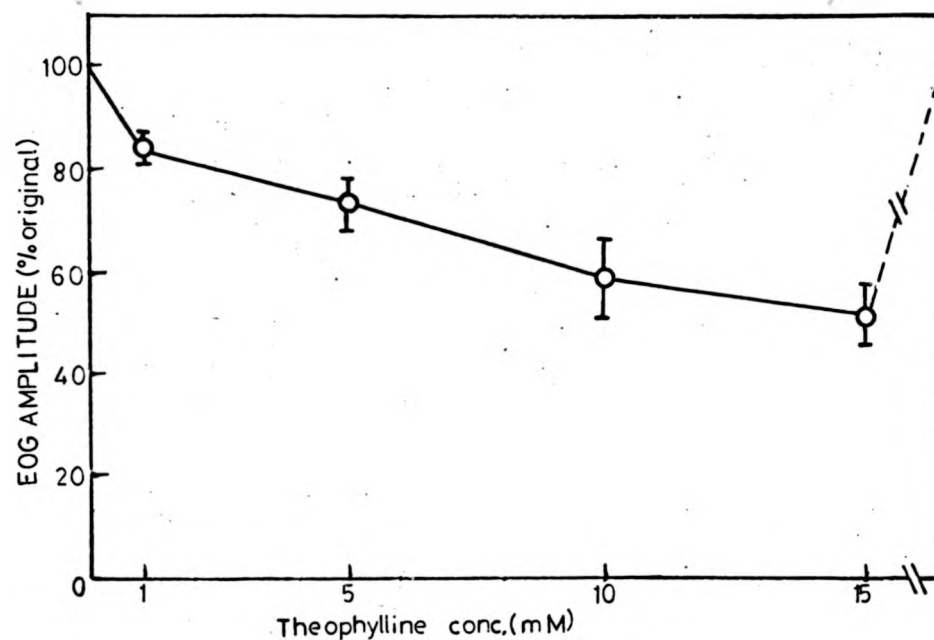
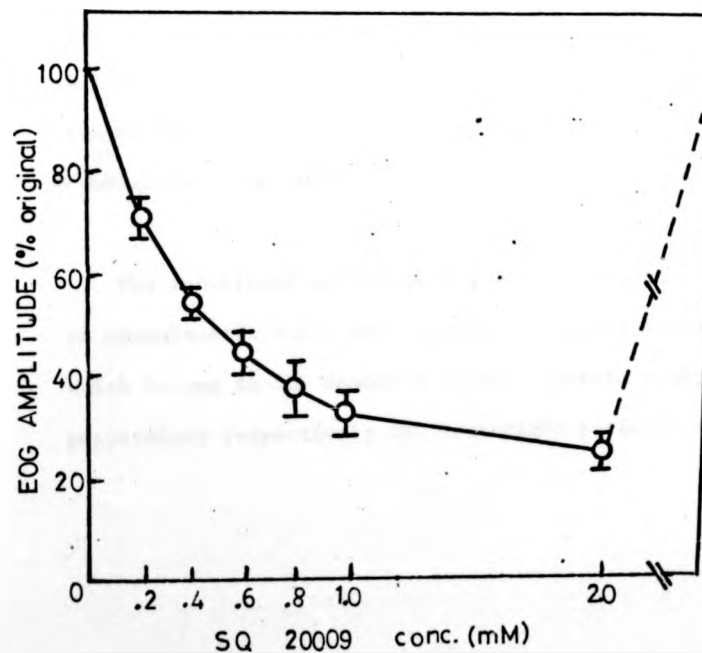


Fig. 69b The effect of SQ 20009 on the EOGs to n-amyl acetate. The broken lines indicate the recovery of the responses after the washing procedure. (2 minutes application of each conc., mean \pm S.E.M. of 6 exp.)



For SQ20009, results are the mean value of 6 runs, those for theophylline of 5 (3 for n-amyl acetate, 2 for ethyl n-butyrate), for caffeine they are for 4, and for RO 20-1724, 3. Bars indicate S.E.M. value of these runs. When the mucosa was washed with frog Ringer's solution after the application of these inhibitors, the EOG response recovers its initial value showing that the effects observed were reversible and were not due to damage to the tissue caused either by high concentration of drugs or by the application of a liquid stimulus. The rank order of the inhibitor concentrations required for a 50% reduction in the EOG amplitude (I_{50}) is the same as that found for other tissues (Free, *et al.*, 1971) and also for soluble phosphodiesterases (Chasin *et al.*, 1972). For the most potent inhibitor, SQ20009, the I_{50} value was 0.51 ± 0.14 mM (mean \pm S.D. of six experiments), which is between one to two orders of magnitude higher than the I_{50} values found for this inhibitor with a range of soluble phosphodiesterases (Chasin *et al.*, 1972). This high value probably reflects the considerable diffusion barrier imposed by the mucus layer which covers the olfactory epithelium. The reduction of the EOG was observed with two floral odorants, ethyl n-butyrate and n-amylacetate, and also with the camphoraceous odorant 1.8 cineole.

The inhibitory action of SQ20009 can be observed on the EOGs of phenylacetic acid, musk ketone, 3-indolyl carbinol and methanol, which belong to the Amoore's class; floral, musky, putrid and pepperminty respectively (S. Cartwright personal publication).

Fig.69c. The effect of theophylline on the EOG responses ethyl n-butyrate
(mean \pm SEM of 2 exp.)

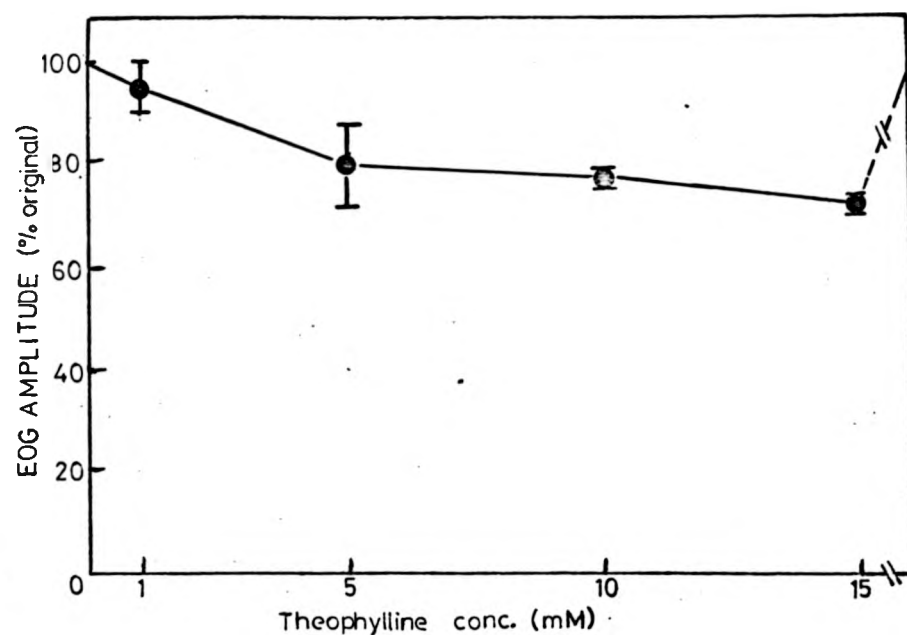


Fig.69d. The effect of Ro20-1724 on the EOGs to n-amyl acetate
(mean \pm SEM of 3 exp.)

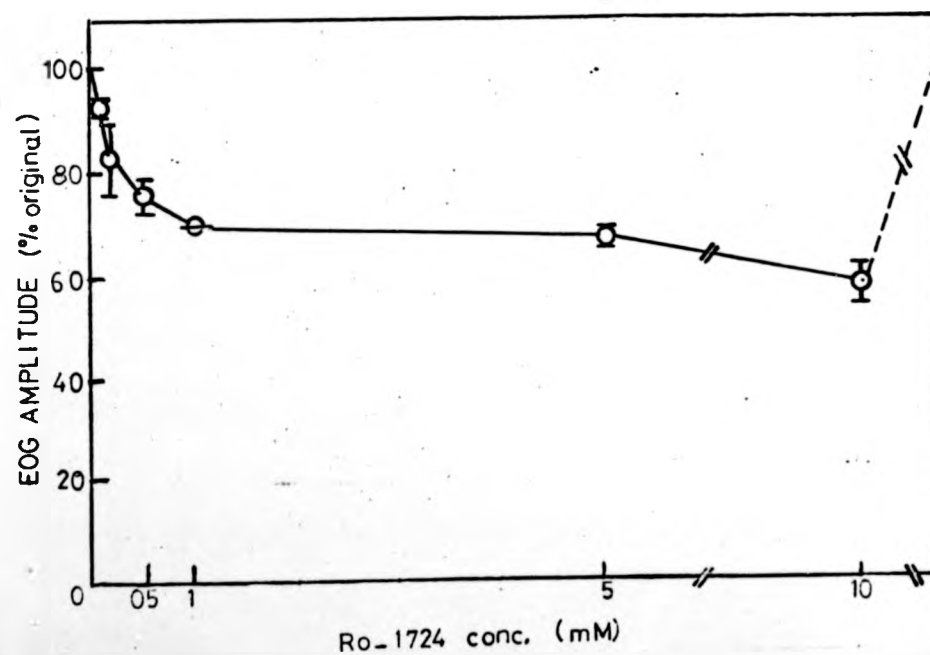
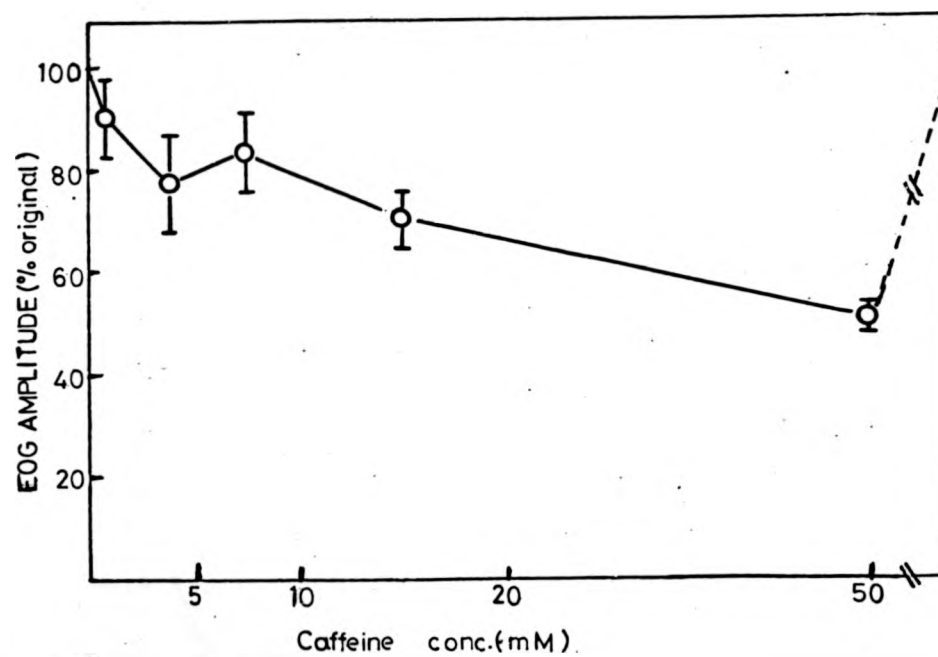


Fig.69e. The effect of caffeine on the EOGs to n-amyl acetate.
(mean \pm SEM of 4 exp)



The effects of the inhibitors develop rapidly. The time course of the inhibition of the EOG amplitudes by phosphodiesterase inhibitors SQ20009 and theophylline is shown in Figure 70.

Exposure of the frog's olfactory mucosa to 10 mM theophylline in Ringer's for 4 minutes reduced the EOG amplitude to ethyl n-butyrate by $37\% \pm 4\%$ (mean \pm S.E.M. of 2 experiments) whereas a $77\% \pm 5\%$ (mean \pm S.E.M. of two experiments) reduction was observed on the EOG amplitude to ethyl n-butyrate after 4 minutes exposure of the olfactory mucosa to 1 mM SQ20009.

After washing the mucosa with frog Ringer's the original EOGs were regained.

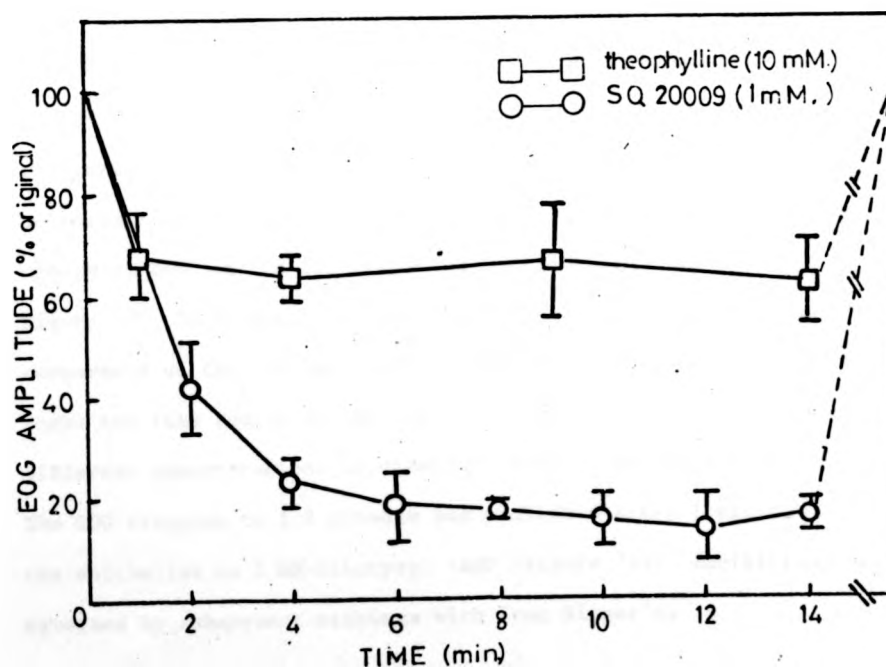


Fig.70 The time course of the inhibition of the EOG amplitudes to ethyl n-butyrate (mean \pm SEM. of 2 exp)

2) EFFECT OF CYCLIC AMP AND ITS MEMBRANE-PERMEABLE DERIVATIVES

After the treatment of olfactory epithelium with cAMP solution in Ringer's, a slight reduction on the EOG peak amplitude was observed. As is seen in Figure 71, the peak was reduced and broadened after the application of 0.1 mM cAMP for 2 minutes. The rising phase of the EOG to n-amyl acetate was not affected but the falling phase was slowed. The "off" response was also not affected. The amplitude of the peak in the EOG response decreased by $17\% \pm 3$ (mean \pm S.E.M. of 2 experiments) after 2 minute application of 0.1 mM cAMP, however, the EOG response did not decrease with an increasing concentration of cAMP (Figure 72). The effect was reversed with frog Ringer's.

The cAMP derivatives N⁶,O^{2'}-dibutyryl-cAMP ("dibutyryl cAMP") and 8-bromo-cAMP, both of which are more permeable to membranes than the parent compound (Hardman *et al.*, 1971) were applied in Ringer's solution to the frog's olfactory mucosa. A typical effect of 1 mM dibutyryl cAMP on the EOG responses to 1,8 cineole is shown in Figure 73. As is seen the time dependent inhibition is on both components of the EOG amplitude, namely peak and plateau. Figure 74 shows the time course of the inhibition of the EOG amplitude by two different concentrations of dibutyryl cAMP, 1 mM and 2 mM respectively. The EOG response to 1,8 cineole was abolished after 1 minute exposure of the epithelium to 2 mM dibutyryl cAMP (Figure 74). Inhibitions were reversed by subsequent washings with frog Ringer's.

Fig.71. Effect of cAMP (0.1 mM for 2 min) on the EOG to n-amy acetate.

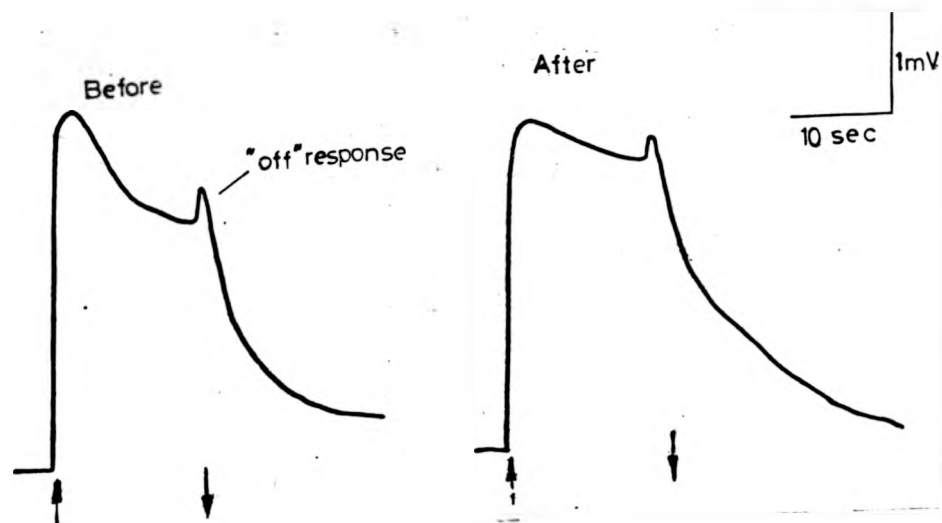


Fig.72 The effects of cAMP on the EOGs to n-amy acetate (mean \pm SEM of 2 exp)

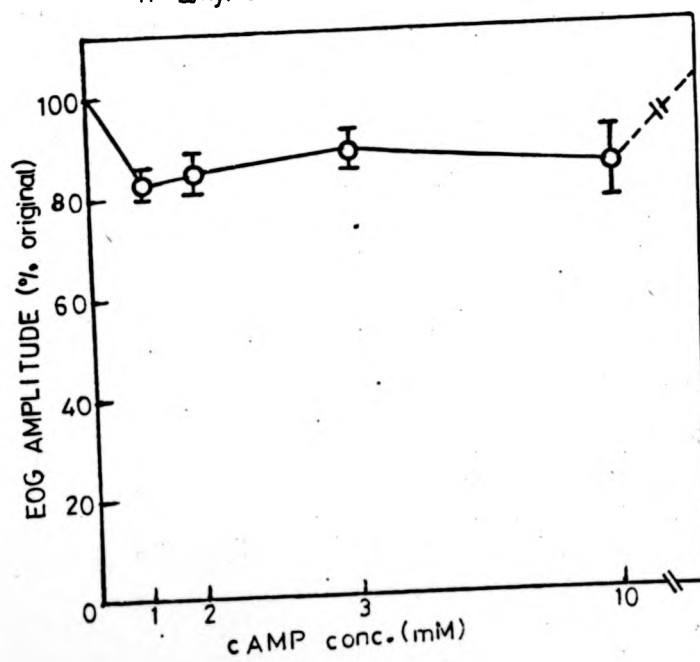
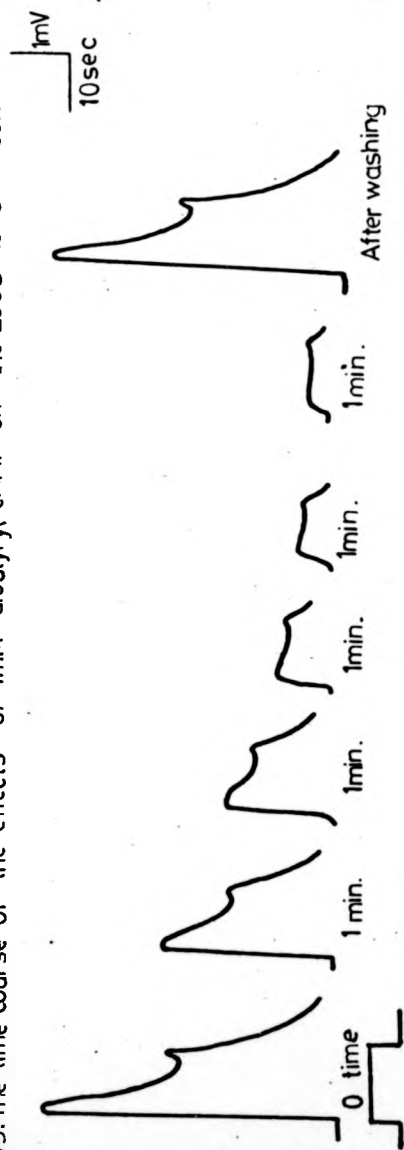


Fig.73. The time course of the effects of 1mM dibutyl cAMP on the EOGs to 18 cinede.



The other cAMP derivative, 8-bromo cAMP, decreased EOG responses in the same fashion as dibutyryl cAMP did. EOG amplitudes to odorants n-amyl acetate and 1,8 cineole were affected. Figure 75 shows the time course of the inhibition of the EOG responses to these odorants, 1,8 cineole being more affected than n-amyl acetate. Again the peak and plateau components were affected in a similar way to the effects of the drug SQ 20009 and dibutyryl cAMP.

Fig.74. The time course of the effects of 1mM and 2mM dibutyl cAMP on the EOGs to 1,8 cineole, (mean \pm SEM. of 2 exp)

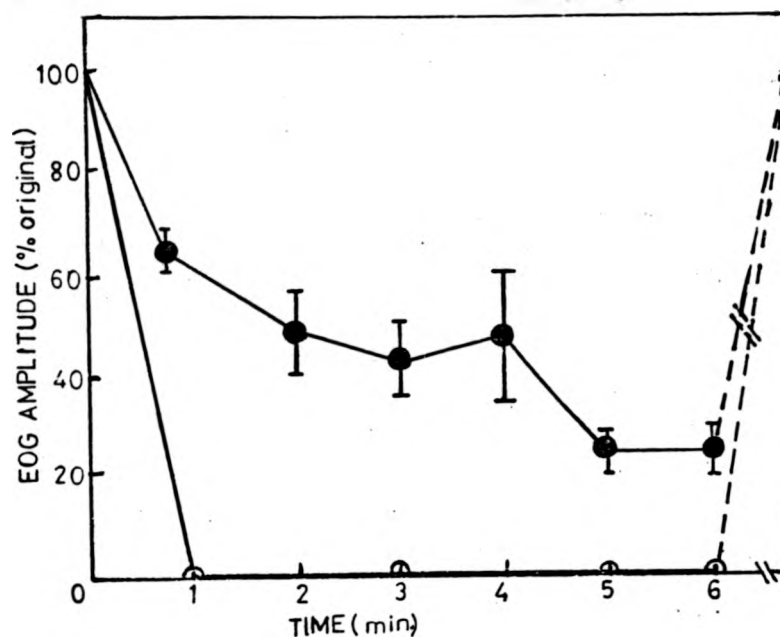


Fig.75. The time course of the inhibition of the EOG amplitude by 8-bromo cAMP(1mM) (mean \pm SEM of 2 exp.)

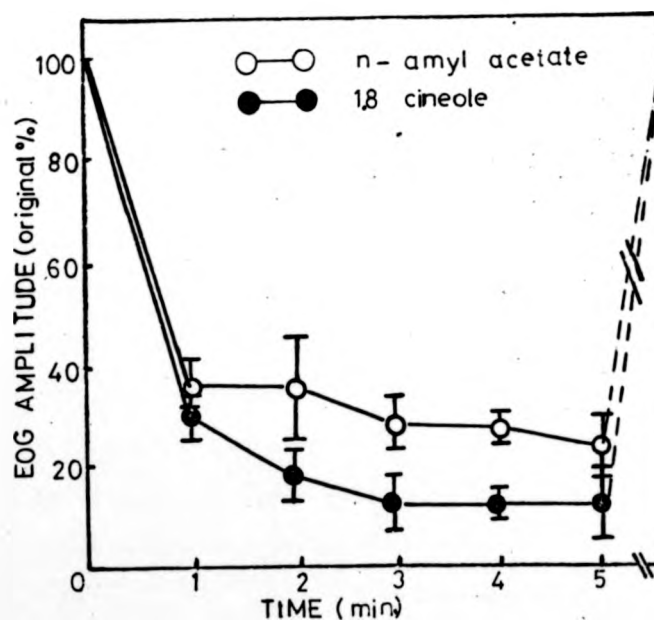
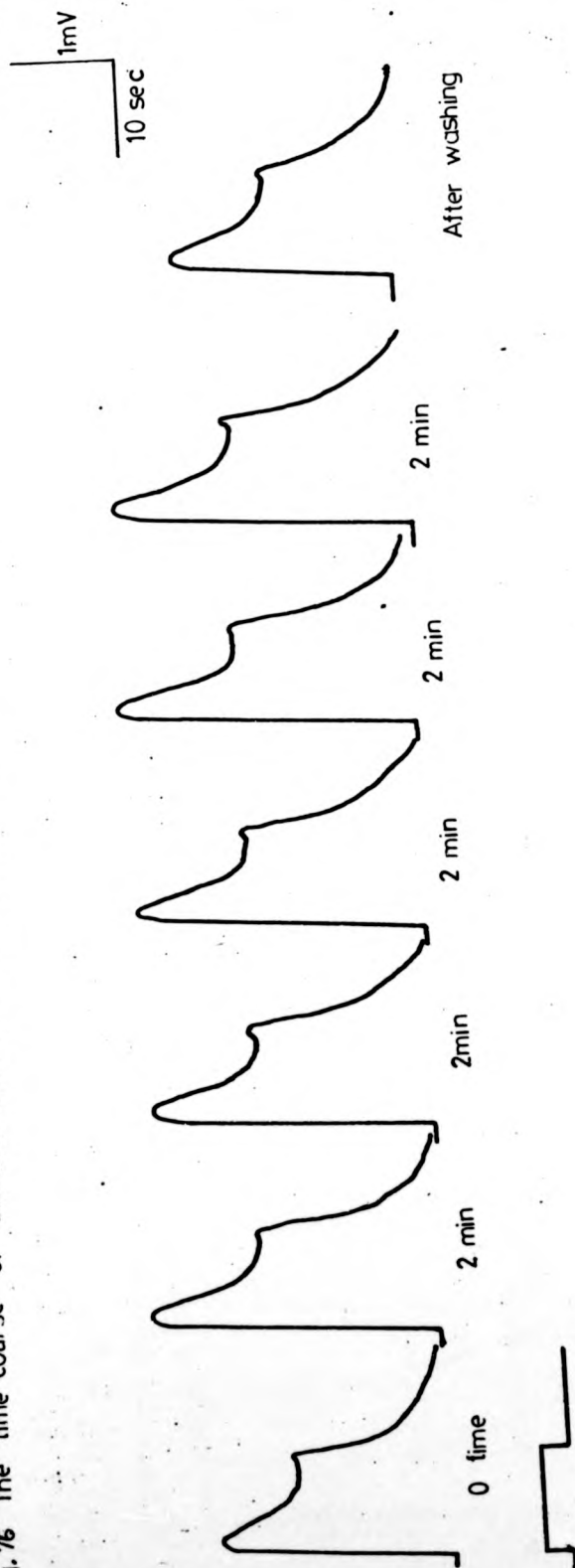


Fig. 76 The time course of the activation of the EOG responses by 40mM Imidazole (odorant is n-amyl acetate)



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3) EFFECT OF OTHER CHEMICALS (AS CONTROLS) AND IMIDAZOLE

A number of chemicals in frog Ringer's solution were applied to the olfactory mucosa. EOG responses to n-amyl acetate and 1,8 cineole were found to be unaffected. Table 3 shows the effect of 2 minutes application of all these chemicals along with the effect of other drugs. The time course of the effect of the control chemicals indicated that up to 15 minutes application of each chemical, the EOGs to the odorant were not affected.

Contrary to phosphodiesterase inhibitors and permeable cAMP derivatives, imidazole increased the EOG amplitude to odorants ethyl n-butyrate and n-amyl acetate. Imidazole was reported to stimulate a phosphodiesterase preparation from rat brain but only at high concentration (Posternak, 1974). It may not be of physiological significance, therefore. However, its time course effect on the EOG responses to n-amyl acetate is seen in Figure 76. The EOG plateau amplitude as well as the peak was increased. The time course of activation of the EOG peak amplitude to ethyl n-butyrate by 40 mM imidazole is shown in Figure 77. The EOG peak amplitude was increased to a maximum of $140\% \pm 3$ (mean \pm S.E.M. of 2 experiments) of the original EOG after the treatment of the mucosa with the drug for 6 minutes. The amplitude of the EOG increases with increasing concentration of the activator and reached a maximum at 40 mM concentration for the odorants n-amyl acetate and ethyl n-butyrate. Further increase in the concentration of the drug (with 100 mM) indicated that there is a big reduction in the activation (Figure 78).

Fig.77. The time course of the activation of EOG responses to ethyl n-butyrate by Imidazole (40 mM) (mean \pm SEM. of 2 exp)

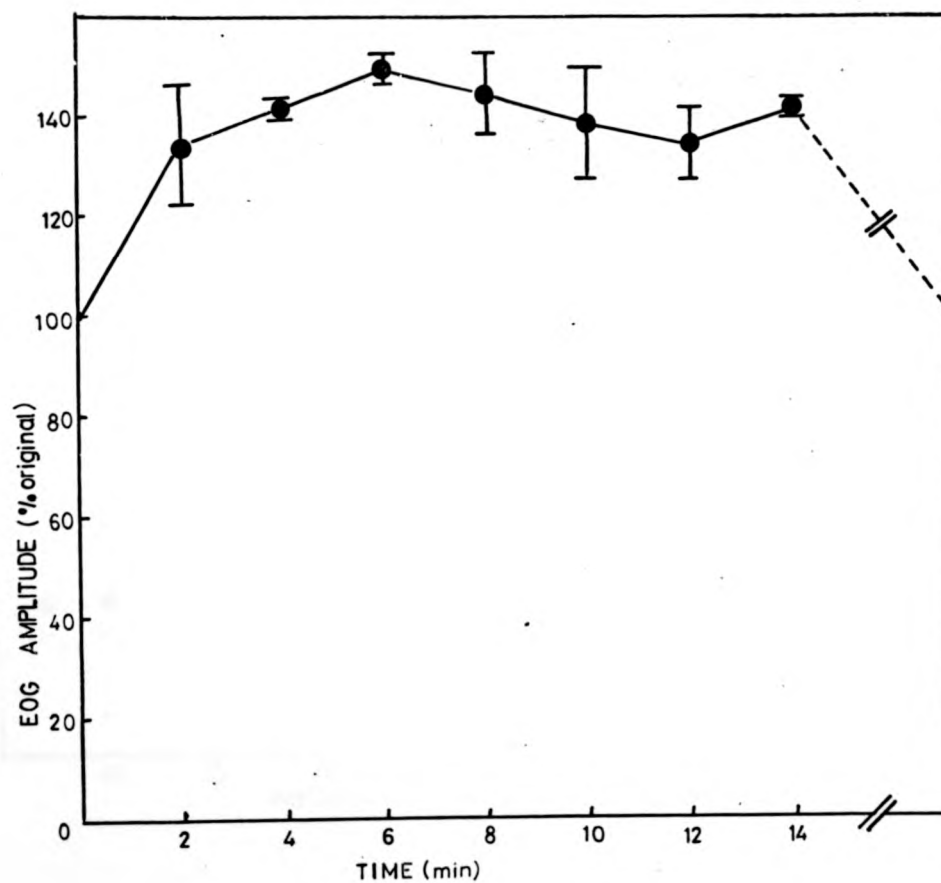


Fig. 78. The effect of imidazole on the EOGs to ethyl n-butyrate (mean \pm S.E.M. of 2 exp.) and n-amyl acetate (mean \pm S.E.M. of 5 exp.)

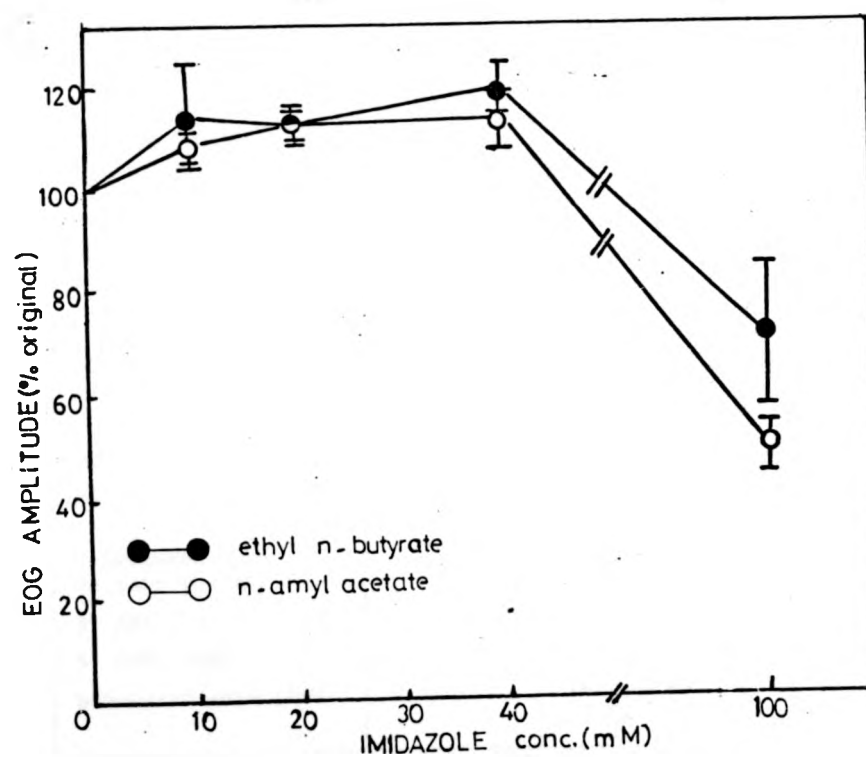


Table 3 . Effect of inhibitors (only 2) and activator of phosphodiesterase
cAMP, cAMP derivatives and other chemicals on the EOG responses
(2 minute applications of each, odour is n-amyl acetate)

| Compound | Increase in response (per cent) | Decrease in response (per cent) | No Effect | S.E.M. |
|---|---------------------------------------|---------------------------------------|--------------------|--------|
| SQ20009 (1mM) | 35 | 58 | | ± 9 |
| Theophylline (10mM) | | 34 | | ± 8 |
| 8-bromo cAMP (1mM) | | 64 | | ±10 |
| dibutyryl cAMP (1mM) (odour is 1,8 cineole) | | 52 | | ± 8 |
| Imidazole (40mM) | | | | ±12 |
| cAMP (0.1mM) | | 17 | | ± 3 |
| ATP (1mM) | | | No effect | |
| GTP (1mM) | | | " | |
| glycine (0.50mM) | | | " | |
| L-phenylalanine (0.50mM) | | | " | |
| cGMP (1mM) | | | " | |
| dibutyryl cGMP (1mM) | | | Very small <10% | |
| L-glutamic acid Na ⁺ salt (0.5mM) | | | | " |
| 5' AMP (1mM) | | | | " |
| 5' GMP (1mM) | | | | " |
| n-butyric acid (1mM) | | | Very small <10% | |

The effects of phosphodiesterase inhibitors, cAMP, cAMP derivatives, imidazole and other chemicals are not associated with injury since they can be completely reversed by washing the mucosa with frog Ringer's solution. The effects can be repeated in the same preparation. Furthermore indifferent molecules such as 5'AMP, cGMP, 5'GMP etc. do not mimic the effects of phosphodiesterase inhibitors, and dibutyryl cAMP or 8-bromo cAMP.

Reduction of the EOG responses to the odorants required a relatively high concentration of the drugs. This may be due to the diffusion barrier imposed by the mucus layer covering the olfactory epithelia. If there is a specific involvement of cAMP in the generation of EOGs, one would expect to observe the changes with low concentrations of the drugs as is normally observed (Chasin *et al.*, 1972). Nevertheless the effect, despite the high concentrations of cAMP, cAMP derivatives and phosphodiesterase inhibitors, is specific (Table 3) within a certain range i.e. indifferent molecules, cGMP, 5'AMP, dibutyryl cGMP etc. do not affect the EOGs.

Further work is required to show the possible involvement of cAMP as an intermediate in the olfactory transduction mechanism. Furthermore, investigations must be conducted to show the presence of all the enzymes involved in the cAMP processes, including protein kinases, and their activity must show changes upon odour-receptor binding.

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